(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(71) Applicant (for all designated States except US): PHAR-MACIA AB [SE/SE]; S-112 87 Stockholm (SE).

(72) Inventors; and
(75) Inventors/Applicants (for US only): ENERBÄCK, Sven [SE/SE]; Slätthultsliden 13 A, S-431 96 Mölndal (SE). CARLSSON, Peter [SE/SE]; Björnbärsvägen 56, S-448 37 Floda (SE).

(74) Agents: HÖGLUND, Lars et al.; Pharmacia AB, Patent Dept., S-112 87 Stockholm (SE).

(54) Title: TRANSGENIC ANIMAL MODEL FOR OBESITY EXPRESSING FOXC2

(57) Abstract: According to the invention, a major role for the winged helix protein FOXC2 in regulating energy balance and adiposity is demonstrated. The invention relates to transgenic non-human mammalian animals being capable of expressing the human FOXC2 gene in its adipose tissue. The invention also relates to methods for identifying compounds useful for the treatment of medical conditions related to obesity or diabetes, said compounds being capable of stimulating expression of the human FOXC2 gene, or being capable of stimulating the biological activity of a polypeptide encoded by the human FOXC2 gene. The invention further relates to methods for identifying compounds useful for the treatment of medical conditions related to malnutrition, said compounds being capable of decreasing expression of the human FOXC2 gene, or being capable of decreasing the biological activity of a polypeptide encoded by the human FOXC2 gene.

TRANSGENIC ANIMAL MODEL FOR OBESITY EXPRESSING FOX C2

TECHNICAL FIELD

5   The invention relates to transgenic non-human mammalian animals being capable of
expressing the human *FOXC2* gene in its adipose tissue. The invention also relates to
methods for identifying compounds useful for the treatment of medical conditions
related to obesity or diabetes, said compounds being capable of stimulating expression
of the human *FOXC2* gene, or being capable of stimulating the biological activity of a
10   polypeptide encoded by the human *FOXC2* gene. The invention further relates to
methods for identifying compounds useful for the treatment of medical conditions
related to malnutrition, said compounds being capable of decreasing expression of the
human *FOXC2* gene, or being capable of decreasing the biological activity of a
polypeptide encoded by the human *FOXC2* gene.

15

BACKGROUND ART

More than half of the men and women in the United States, 30 years of age and older,
20   are now *considered* overweight, and nearly one-quarter are clinically obese
(Wickelgren, 1998). This high prevalence has led to increases in the medical conditions
that often accompany obesity, especially non-insulin dependent diabetes mellitus
(NIDDM), hypertension, cardiovascular disorders, and certain cancers. Perhaps most
importantly, obesity confers a significant increased rate of mortality when compared
25   with that of individuals of normal body weight. Obesity results from a chronic
imbalance between energy intake (feeding) and energy expenditure. Energy expenditure
has several major components including basal metabolism, physical activity, and
adaptive (nonshivering) thermogenesis. This latter process refers to energy that is
dissipated in response to changing environmental conditions, most notably exposure to
30   cold or excessive caloric intake (so-called diet-induced thermogenesis). To better
understand the mechanisms that lead to obesity and to develop strategies in certain
patient populations to control obesity, we need to develop a better underlying
knowledge of the molecular events that regulate the differentiation of preadipocytes and
stem cells to adipocytes, the major component of adipose tissue.

Role of adipose tissue

The reason for existence of the adipocyte is to store energy for use during periods of caloric insufficiency. Postprandially, dietary fat is absorbed via the intestine and secreted into the circulation as large triglyceride (TG) rich particles called chylomicrons (chylo). Lipoprotein lipase (LPL), although produced by adipocytes, is localized to the endothelial cell surface where it hydrolyses TG resulting in the release of free fatty acids (FFA). Much of these are taken up by the adipose tissue either passive or active via FFA transporters. The FFAs are then activated to an acyl CoA form and re-esterified by an enzymatic cascade to form storage TG. At the same time, glucose, which also increases in the circulation postprandially, is taken up into adipose tissue via specific plasma membrane glucose transporters. These two substrates (glucose and FFA) are the building blocks for formation of storage TG. On the other hand, during fasting, FFAs are released from the adipose tissue TG pool through the action of hormone sensitive lipase (HSL; Fig. 1). Clearly, efficient functioning of adipose tissue is dependent on the coordinated control of each of these processes and the proteins involved.

In recent years, a growing body of evidence has demonstrated a dual role for adipocytes, also being a source of numerous hormones that regulate both the adipocyte itself and many other systems within the body. Adipocytes produce leptin as a function of adipose energy stores. Leptin acts through receptors in the hypothalamus to regulate appetite, activity of brown adipose tissue (BAT), insulin secretion via sympathetic nervous system output, and important neuroendocrine adaptive responses to fasting and control of reproduction. The gene encoding leptin was identified by positional cloning (Zhang *et al.*, 1994) and is the mutation leading to the profound obese phenotype of the ob/ob mouse, characterized by severe obesity, NIDDM, diminished fertility and hypothermia. The db-gene codes for a hypothalamic receptor for leptin (Chua *et al.*, 1996) and the db/db mutant mice show a similar phenotype with ob/ob mice, but here the defect lies in the block of leptin receptor downstream signaling. After leptin administration, it was possible to correct the defect only in the ob/ob, but not db/db mice as predicted by Coleman's parabiosis experiments (Coleman, 1973).

Another adipocyte product, the cytokine tumor necrosis factor α (TNFα), has profound effects on adipocyte differentiation, and energy metabolism, and can even induce adipocyte dedifferentiation and apoptosis. Furthermore, TNFα has more systemic implications as it has been shown to play a role in the genesis of insulin resistance

5   associated with obesity (Hotamisligil *et al.*, 1993). In obese humans and numerous rodent models of obesity-diabetes syndromes, there is a marked elevation in muscle and adipose TNFα production, as compared with tissues from lean individuals (Hotamisligil *et al.*, 1995; Hotamisligil *et al.*, 1993). TNFα levels can be reduced with weight loss (Hotamisligil *et al.*, 1995) or after treatment with the insulin-sensitizing agent

10   pioglitazone (Hofmann *et al.*, 1994).

A third adipocyte product, the acylation stimulating protein (ASP) exert autocrine action on the adipocyte, having potent anabolic effects on human adipose tissue by stimulation of glucose transport and FFA esterfication (Maslowska *et al.*, 1997; Walsh

15   *et al.*, 1989). ASP is generated by the interaction of complement D (identical to adipsin), factor B, and complement C3, components of the alternate complement pathway all produced by adipocytes (Choy and Spiegelman, 1996).

White adipose tissue versus brown adipose tissue

20

There are two different types of adipose tissue in the body, WAT and BAT, which have quite opposite physiological functions although they both have the same "machinery" for lipogenic and lipolytic activity. WAT stores excess energy as triglycerides and releases free fatty acids in response to energy requirements at other sites. BAT on the

25   other hand is involved in adaptive (non-shivering) thermogenesis. BAT is found only at certain sites in the body of rodent, such as in interscapular, perirenal and retroperitoneal regions. In human neonates BAT is present in large quantities but its thermogenic activity decreases shortly after birth and the tissue is gradually converted into white type adipose tissue (Lean *et al.*, 1986). However, judged by expression of the brown fat

30   specific uncoupling protein 1 (UCP1) mRNA, substantial amounts of brown adipocytes exist throughout life in human adipose deposits, which are generally classified as white (Krief *et al.*, 1993).

3

Brown adipocytes have a multilocular disposition of fat droplets, i.e. a number of individual droplets within each adipocyte, whereas the white adipocyte has a single fat droplet within the cell. Furthermore, the brown adipocyte has a central nucleus and a large number of mitochondria in contrast to the white adipocyte, which has very few

5   mitochondria and a nucleus that is displaced towards the plasma membrane by the lipid droplet. The only known gene marker to distinguish BAT from WAT, or any other celltypes is the expression of UCP1 in brown adipocytes. Due to the presence of this unique mitochondrial protein brown adipocytes have the ability of facultative heat production, which is highly regulated by sympathetic nerve activity. UCP1 is a proton

10   translocator in the inner mitochondrial membrane and functions as a facultative uncoupler of the mitochondrial respiratory chain (Nicholls and Locke, 1984). Recently two new uncoupling proteins have been identified and cloned through their sequence homology with UCP1. UCP2 is found in most tissues (Fleury *et al.*, 1997), while UCP3 is expressed in BAT and skeletal muscle (Boss *et al.*, 1997). The respective roles for

15   UCP2 and UCP3 in thermogenesis and energy balance of intact animals remain to be determined. That brown fat is highly important in rodents for maintaining nutritional homeostasis is predicted by the facts that the function of BAT is impaired in obese rodents (Himms-Hagen, 1989) and transgenic mice with decreased brown fat mass develop obesity (Lowell *et al.*, 1993). Since BAT is much less obvious in large animals

20   like humans, than in rodents, skeletal muscle is thought to be the site of primary importance for normally occurring adaptive thermogenesis in large animals.

Both white and brown fat are innervated under the control of the sympathetic nervous system. There are at least three pharmacologically distinct subtypes of β-adrenergic

25   receptors (β_1, β_2, and β_3) found in adipocytes. The β_3-adrenergic receptor (β_3-AR) is the predominant subtype in adipose tissue and it mediates the effects of norepinephrine present in the sympathetic synaptic cleft during nerve stimulation of lipolysis in WAT and BAT and of thermogenesis in BAT (Giacobino, 1995). Increased lipolysis takes place primarily through the production of cAMP and the activation of hormone-

30   sensitive lipase through phosphorylation (Fig. 1). Thermogenesis in BAT is accomplished by increased UCP1 mRNA levels through stimulation of transcription (Rehnmark *et al.*, 1990; Ricquier *et al.*, 1986). Uncoupled respiration is also thought to be stimulated by increased lipolysis and the raise in intracellular concentration of FFA

(Jezek *et al.*, 1994). Sympathetic stimulation of brown fat also contributes to regulation of energy expenditure by increasing mitochondrial biogenesis (Wu *et al.*, 1999a) and hyperplasia of brown adipocytes. In rodents, β_3-adrenergic receptors (β_3-ARs) are abundant in WAT and BAT (Granneman *et al.*, 1991; Muzzin *et al.*, 1991; Nahmias *et al.*, 1991), while in humans, β_3-AR mRNA is abundant in BAT only, with much less or no β_3-AR mRNA found in WAT (Granneman and Lahners, 1994; Krief *et al.*, 1993). Long-term treatment of obese rodents with β_3-selective agonists reduces fat stores and improves obesity-induced insulin resistance (Bloom *et al.*, 1992; Cawthorne *et al.*, 1992; Holloway *et al.*, 1992). Thus, β_3-selective agonists are promising anti-obesity compounds. Trials of β_3-AR agonist treatment, aimed at stimulating BAT in humans have proved disappointing with respect to weight loss (Arch and Wilson, 1996). The true potential of β_3-AR agonists in humans can only be evaluated when a compound with good selectivity and efficacy at the human β_3-AR, coupled with a long duration of action *in vivo*, has been identified, however those compounds that have been evaluated in humans so far have much lower efficacy at the human than the rodent receptor. This could be explained by the fact that human and mouse/rat β_3-AR show a ~80% similarity in their amino acid sequence. Several of the β_3-AR-selective agonists (e.g. BRL 37344 and CL 316,243) have been shown to be extremely potent against mouse and rat β_3-AR but with a greatly reduced activity against the human β_3-AR. Presently, recombinant cell lines expressing human β_3-ARs are being used to identify compounds with an increased potency against the human receptor (Ito *et al.*, 1998). Mice with targeted mutagenesis of the β_3-AR gene show only a modest tendency to become obese and their brown fat response to cold exposure works perfectly normal (Susulic *et al.*, 1995). Deficient mice displayed an up-regulation of β_1-AR mRNA levels in both white and brown fat which most probably is the reason of the mild phenotype. These results implicate that it is possible that β_1- and β_2-ARs also play important roles in innervation of adipose tissue. Moreover, other species, including humans, have higher levels of β_1- and β_2-ARs, then β_3-AR, in adipose tissue (Lafontan and Berlan, 1993).

Adipocyte differentiation

There has been some great progress during the past few years in the understanding of the adipocyte differentiation program. Most of the work leading to this understanding has been carried out using white preadipose cell lines in culture, notably the C3H10T½ and NIH 3T3 fibroblastic cell lines and the 3T3-L1 and 3T3-F442A preadipocyte cell lines. Treatment of multipotent C3H10T½ cells with 5-azacytidine (a demethylating agent) gives rise to cells committed to the myogenic, adipogenic, osteoblastic, or chondrogenic lineages. This is consistent with the view that the adipose lineage arises from the same multipotent stem cell population of mesodermal origin that gives rise to the muscle and cartilage lineages (Cornelius *et al.*, 1994). Under appropriate hormonal control (e.g. glucocorticoid, insulin-like growth factor-1, and cyclic AMP or factors that mimic these agents) or experimental manipulation white preadipose cell lines are capable to differentiate into mature white adipocytes (Ailhaud *et al.*, 1992). Several transcription factors have been identified, which act co-operatively and sequentially to trigger the functional differentiation program (Fig. 2).

Transcriptional control of adipocyte differentiation through PPARs C/EBPs, and ADD1/SREBP1

Peroxisome proliferator-activated receptors (PPARs) are a class of the nuclear hormone receptors. The member PPARγ is now well recognized as serving an important role in the regulation of adipogenesis. Through the use of alternate promoters, the gene encoding PPARγ gives rise to two separate products, PPARγ1 and PPARγ2, the latter containing an additional 28 N-terminal amino acids that are reported to enhance ligand binding (Fajas *et al.*, 1997; Werman *et al.*, 1997). Reports that ligand activation of retrovirally expressed PPARγ2 in non-differentiating NIH-3T3 cells potently promoted adipocyte differentiation provided the most compelling evidence for the adipogenic nature of PPARγ2 (Tontonoz *et al.*, 1994b). One live-born PPARγ deficient mouse has been produced and it displayed a total absence of WAT and BAT (complete lipodystrophy) and fatty liver, secondary to lipodystrophy (Barak *et al.*, 1999).

Members of the PPAR family specifically function as heterodimers with the retinoid X receptor (RXR) through interactions with peroxisome proliferator response elements (PPREs) on target genes, including lipoprotein lipase (LPL; Schoonjans et al., 1996), the adipocyte fatty acid-binding protein 422/aP2 (Tontonoz et al., 1994a),

5   phosphoenolpyruvate carboxykinase (PEPCK; Tontonoz et al., 1995), and stearocyl-CoA desaturase 1 (Miller and Ntambi, 1996). Transcriptional activity of PPARγ is induced following binding of either synthetic or naturally occurring ligands, including prostaglandins of the D2 and J2 series, with the 15-deoxy-Δ 12, 14-prostaglandin J2 derivate emerging as one of the most potent (Forman et al., 1995). Synthetic ligands

10   that activate PPARγ include carbacyclin and a new class of antidiabetic drugs, the thiazolidinediones (TZDs) (Lehmann et al., 1995). TZDs promote adipogenesis in culture and improve insulin sensitivity in vivo. PPARγ activators probably modify the production of adipocyte-derived mediators of insulin resistance, such as free fatty acids or TNFα. PPARγ activation will decrease production of TNFα by adipocytes and

15   interfere with its inhibitory effect on insulin signaling (Peraldi et al., 1997).

In addition, because of its tissue selective effects on genes involved in fatty acid uptake, PPARγ activation will induce repartitioning of fatty acids in the body, with enhanced accumulation of fatty acids in adipose tissue at the expense of a relative depletion of

20   muscle fatty acids (Martin et al., 1997). The relative lipid depletion of muscle cells will improve their glucose metabolism and result in an improvement in insulin sensitivity. Furthermore, PPARγ decreases the expression of the adipocyte-derived signaling molecule leptin, which results in an increase in energy intake and optimization of energy usage, contributing further to PPARγ's adipogenic effect (De Vos et al., 1996).

25   Recently it has been demonstrated that interaction with a novel cofactor PPARγ coactivator (PGC-1), could enhance PPARγ transcriptional activity in brown adipose tissue (Puigserver et al., 1998).

Three members of the CCAAT/enhancer-binding protein (C/EBP) family of

30   transcription factors, i.e. C/EBPα, C/EBPβ, and C/EBPδ, have been implicated in the induction of adipocyte differentiation. The factors are proteins of the bZIP class, with a basic domain that mediates DNA binding and a leucine zipper dimerization domain. Cyclic AMP and adipogenic hormones such as glucocorticoids and insulin induce a

7

transient increase in the expression of C/EBPβ and δ early in adipocyte differentiation (Cao *et al.*, 1991; MacDougald *et al.*, 1994; Yeh *et al.*, 1995). C/EBPβ, in synergy with C/EBPδ, then induces PPARγ expression in the preadipocyte (Wu *et al.*, 1996, Wu *et al.*, 1995). Mice lacking the C/EBPβ, and C/EBPδ gene have normal expression of

5   C/EBPα and PPARγ, but this co-expression of C/EBPα and PPARγ is not sufficient for complete adipocyte differentiation in the absence of C/EBPβ and C/EBPδ (Tanaka *et al.*, 1997). C/EBPα seems to play an important part in the later stages of differentiation by maintaining the differentiated adipocyte phenotype through autoactivation of its own gene (Lin and Lane, 1992; Lin and Lane, 1994). C/EBPα activates several adipocyte-

10   specific genes such as the insulin-responsive glucose transporter-4 (GLUT4) (Kaestner *et al.*, 1990), 422/aP2 (Christy *et al.*, 1989), UCP1 (Yubero *et al.*, 1994), and also the insulin receptor gene, and insulin receptor substrate 1 (IRS-1) (Wu *et al.*, 1999b). Definitive proof that C/EBPα is required for adipocyte differentiation was obtained by showing that expression of antisense C/EBPα RNA in 3T3-L1 preadipocytes prevented

15   differentiation (Samuelsson *et al.*, 1991). Consistent with this finding, disruption of the C/EBPα gene gave rise to mice that failed to develop white adipose tissue (Wang *et al.*, 1995). Taken together these findings proved that C/EBPα is both required and sufficient to induce adipocyte differentiation. The expression of C/EBPα, as well as other adipocyte genes, is induced upon ligand activation of PPARγ. Through a positive

20   feedback loop, C/EBPα maintains the expression of PPARγ. C/EBPα and PPARγ cooperate to promote adipocyte differentiation, including adipocyte gene expression and insulin sensitivity (Wu *et al.*, 1999b). It is possible that C/EBPα is ultimately an important, indirect target of the antidiabetic actions of the TZDs.

25   ADD1/SREBP1 (adipocyte determination and differentiation-dependent factor 1/sterol regulatory element binding protein 1) is a member of the basic helix-loop-helix (bHLH) class of transcription factors. In the inactive state, the protein is membrane-bound to the endoplasmic reticulum. Upon activation (such as a low cholesterol state), ADD1/SREBP1 is proteolytically cleaved and the soluble form becomes translocated to

30   the nucleus where it binds one of two different response elements, namely the E box and the sterol regulatory element (SRE; Brown and Goldstein, 1997). The expression of ADD1/SREBP1 is induced during differentiation of adipocytes, where it activates

β

transcription of target genes involved in both cholesterol metabolism and fatty acid metabolism (Kim and Spiegelman, 1996). ADD1/SREBP1 potentiates the transcriptional activity of PPARγ probably through the production of endogenous ligands for PPARγ (Kim *et al.*, 1998) and also by binding to and inducing the PPARγ promoter (Fajas *et al.*, 1999).

When preadipocytes differentiate into adipocytes, several differentiation-linked genes are activated. Lipoprotein lipase (LPL) is one of the first genes induced during this process (Fig. 2). Two cis-regulatory elements important for gradual activation of the LPL gene during adipocyte development *in vitro* have been delimited (Enerback *et al.*, 1992). These elements, LP-α and LP-β, contained a striking similarity to a consensus sequence known to bind transcription factors of the winged helix family. Results of gel mobility shift assays and DNase I and exonuclease III *in vitro* protection assays indicated that factors with DNA-binding properties similar to those of the winged helix family of transcription factors are present in adipocytes and interact with LP-α and LP-β. There is a need for identifying human winged helix genes that could be responsible for the induction of the LPL promoter and possibly regulating expression of other adipocyte specific genes.

"Fork head" and "winged helix" genes

The "fork head" domain is an evolutionary conserved DNA-binding domain of 100 amino acids, which emerged from a sequence comparison of the transcription factor HNF-3α of rat and the homeotic gene *fork head* of *Drosophila*. X-ray crystallography of the fork head domain from HNF-3γ revealed a three-dimensional structure, the "winged helix", in which two loops (wings) are connected on the C-terminal side of the helix-loop-helix (for reviews, see Brennan, R.G. (1993) Cell 74, 773-776; Lai, E. et al. (1993) Proc. Natl. Acad. Sci. U.S.A. 90, 10421-10423).

The isolation of the mouse mesenchyme forkhead-1 (MFH-1) and the corresponding human (*FKHL14*) chromosomal genes is disclosed by Miura, N. et al. (1993) FEBS letters 326: 171-176; and (1997) Genomics 41: 489-492. The nucleotide sequences of the mouse MFH-1 gene and the human *FKHL14* gene have been deposited with the

EMBL/GenBank Data Libraries under accession Nos. Y08222 (SEQ ID NO: 5) and
Y08223 (SEQ ID NO: 8), respectively. A corresponding gene has been identified in
Gallus gallus (GenBank accession numbers U37273 and U95823).

5   The International Patent Application WO 98/54216 discloses a gene encoding a
Forkhead-Related Activator (FREAC)-11 (also known as S12), which is identical with
the polypeptide encoded by the human *FKHL14* gene disclosed by Miura, *supra*.

The nomenclature for the winged helix / forkhead transcription factors has been
10   standardized and Fox (Forkhead Box) has been adopted as the unified symbol (Kaestner
et al. (2000) Genes & Development 14: 142-146; see also
htpp://www.biology.pomona.edu/fox). It has been agreed that the genes previously
designated MFH-1 and *FKHL14* (as well as FREAC-11 and S12) should be designated
FOXC2.

15

DESCRIPTION OF THE DRAWINGS

FIGURE 1
20   Schematic view of lipogenic and lipolytic actions in the adipocyte. Triglycerides are
hydrolyzed to glycerol and free fatty acids by the action of lipoprotein lipase. FFAs are
transported into the adipocyte via FATP. FFAs are combined with acetyl CoA to
produce acyl CoA, which is re-esterfied to triglycerides. The multi-enzyme complex
hormone sensitive lipase hydrolyses triglycerides into FFAs and glycerol. FFAs can
25   either be re-esterfied again or be released into the circulation. LPL, lipoprotein lipase;
FFA, free fatty acid; FATP, fatty acid transport protein; aP2, adipocyte fatty acid-
binding protein 422/aP2; ACS, acyl CoA synthetase; Glut4, glucose transporter IV;
βAR, β-adrenergic receptor; AC, adenylate cyclase; PKA, protein kinase A; HSL,
hormone sensitive lipase. Figure is adapted from Sethi and Hotamisligil, 1999.

30

FIGURE 2
Summary of different stages and events during *in vitro* adipocyte differentiation. Our
current understanding of adipocyte differentiation indicates that a pluripotent stem cell

precursor gives rise to a mesenchymal precursor cell (multipotent) with the potential to differentiate along mesoderma linages of myoblast, chondroblast, osteoblast, and adipocyte. Given appropriate environmental and gene expression cues, preadipocytes undergo clonal expansion and subsequent terminal differentiation. Selected molecular

5   events accompanying this process are indicated above. Pref-1, preadipocyte factor 1; pOb24/A2COL6, α2 chain of type VI collagen; LPL, lipoprotein lipase; aP2, adipocyte fatty acid-binding protein 422/aP2; UCP, uncoupling protein; CUP, C/EBPα undifferentiated protein; FAAR, fatty acid-activated receptor; PPARγ, peroxisome proliferator-activated receptor γ; C/EBP, CCAAT/enhancer binding protein; RXR,

10   retinoid X receptor. Figure is adapted from Klaus, 1997.

FIGURE 3

Northern blot analysis. Adult human tissues (a) and wt adult mouse tissues (b). The blots were analysed with probes specific (with no cross-reactivity) for *FOXC2* (human)

15   and *FoxC2* (mouse), respectively. The experiments were carried out with 20 µg of total RNA/lane using β-actin as an internal control. Mouse adipose tissue (c) was treated with collagenase (see under "Methods"), RNA from the adipocyte fraction (Adip F) and the stroma/vascular fraction (SVF) were used and probed for *FoxC2*, LPL and GAPDH (control). In (d) 3T3-L1 cells were differentiated to adipocytes in culture and then

20   incubated for 6 hours in the absence (Basal) or presence of forskolin (Forsk.; 100 µM), 12-O-tetradecanoylphorbol-13-acetat (TPA; 100 nM), Ca^{2+} ionophore (A23187; 0.5 µM) alone or in combination as indicated. Northern blot analysis was performed and the *FOXC2* mRNA signal was assessed by β-scintillation counting in a dot-matrix β-counter. Image shows counts over the filter plotted in gray-scale intensity. Bars above

25   the Northern blot represent actual cpm over mean cpm of controls subtracted of background cpm given as "fold induction".

FIGURE 4

Schematic view of *FOXC2* transgene construct and Southern blot detection of

30   integration positive mice. A 2.1-kb fragment containing the *FOXC2* cDNA was ligated downstream of the 5.4-kb aP2 enhancer/promoter region (A). Prior to pronuclear injection the transgene construct was liberated from the plasmid as an 8.9-kb *NotI/AgeI* fragment. A 1.7-kb fragment of the aP2 promoter was used as a probe in Southern blot

analysis for identification of integration positive mice. Panel B shows the identification
of the three founders investigated further.

FIGURE 5

(a): Weights of intra-abdominal WAT and interscapular BAT depots from tg mice
(founder lines Tg-A, Tg-B and Tg-C) compared to that of wt mice. (b): Ratios between
weights of intra-abdominal WAT to interscapular BAT for wt and tg founder lines A, B
and C. Values are means ±SEM, n=4 in each group. (c): Expression levels of the
FOXC2 transgene in white adipose tissue (WAT) and brown adipose tissue (BAT) of
wild-type mice (WT) and FOXC2 transgenic mice (founder A, B, and C) as measured
by Northern blot analysis (12 μg of total RNA/lane). GAPDH was used as a control.

FIGURE 6

A 5-months-old wild-type female and a FOXC2 transgenic female littermate. A, C, and
E show WT; B, D, and F show transgenic littermate. (A, B) An exposed dorsal view of
the interscapular brown fat pads, illustrating an increased size of the depot in the
transgenic mouse. (C, D) An exposed ventral view, illustrating a reduction in size and
change in appearance of the intrabdominal white fat pads in the transgenic mouse. (E,
F) The interscapular brown fat pads and the intrabdominal white fat pads have been
dissected out. BAT at the top and WAT at the bottom.

FIGURE 7

Histologic sections of brown fat (A, B), and white fat (C, D) from a 5-months-old wild-
type mouse (left) and a FOXC2 transgenic littermate (founder A) (right). (A, B)
Interscapular brown fat of the transgenic mouse consists of markedly enlarged
adipocytes containing large unilocular fat droplets. (C, D) Adipocytes in white fat of the
transgenic mouse show heterogeneity of size. This is in contrast to the WAT from a
wild-type mouse, which consists of adipocytes of uniform size filled with a large,
unilocular vacuole. In a cold adaptation experiment, mice were kept at either room
temperature (RT) or at 4°C for 24 hours. Whereas there is no apparent difference
between BAT from wt mice (E, G), the number and size of lipid droplets present in tg
BAT at room temperature is clearly reduced when exposed to 4°C (F, H). Scale bar
depicted in G equals 100 μm.

12

FIGURE 8

Change of size of fat depots in *FOXC2* transgenic mice (founder C). (A) Weight
comparison (expressed as percent of total body weight) for intrabdominal fat depots and
5   interscapular brown fat depots of 5-months-old wild-type females and *FOXC2*
transgenic littermates. Changes are significant, $P<0.005$. (B) Ratio between weights of
the intrabdominal fat depot and the interscapular brown fat depot. Change is significant,
$P<0.0005$. (C) No significant difference in body weight could be detected between the
two groups. (D) No significant difference in food consumption was noticed when
10   measured during a time period of two months. Data are means ±SD, n=3 for both WT
and founder C in A-C, n=4 for both WT and founder C in D.

FIGURE 9

Amount of different mRNAs in white adipose tissue (WAT) and brown adipose tissue
15   (BAT) of wild-type mice (WT) and *FOXC2* transgenic mice (founder A, B, and C) as
measured by Northern blot analysis (12 µg of total RNA/lane). GAPDH was used as a
control for ensuring equal loading on all blots.

FIGURE 10

20   Serum triglyceride is lowered in *FOXC2* transgenic mice (founder A). Serum
triglyceride content was analyzed for 14-weeks-old wild-type males and *FOXC2*
transgenic littermates fed ad libitum. Change is significant, $P<0.005$. Data are means
±SD, n=4 for both WT and founder A.

25   FIGURE 11

Total body lipid content is lowered in *FOXC2* transgenic mice (founder A). Total body
lipid content was analyzed for 6-months-old wild-type males and *FOXC2* transgenic
littermates fed ad libitum. Change is significant, $P<0.0005$. Data are means ±SD; WT,
n=3 and founder A, n=4.

30

FIGURE 12

Blood glucose is lowered (A) and glucose elimination is more efficient (B) in *FOXC2*
transgenic mice (founder A). (A) Blood glucose levels were analyzed for 10-weeks-old

wild-type mice and *FOXC2* transgenic littermates fed ad libitum. A significant (P<0.05) reduction of blood glucose levels were seen for *FOXC2* transgenic mice. (B) An intravenous glucose tolerance test was carried out on the mice used in (A). Blood samples were taken immediately before and at 1, 5, 20, and 50 min after intravenous

5   injection of glucose (1 g/kg). The values were significantly changed at 0 min (P<0.05), 20 min (P<0.001), and at 50 min (P<0.05). Data are means ±SD; n=10 for both WT and founder A.

FIGURE 13

10 Plasma insulin is lowered in *FOXC2* transgenic mice (A), still levels raised higher than in wild-type after intravenous (iv) glucose load (B, C) (founder A). (A) Plasma insulin levels were analyzed for 10-weeks-old wild-type mice and *FOXC2* transgenic littermates fed ad libitum. A significant (P<0.05) reduction of plasma insulin levels was seen for *FOXC2* transgenic mice. (B) An intravenous glucose tolerance test was carried

15 out on the mice used in (A) Blood samples were taken immediately before and at 1, 5, 20, and 50 min after intravenous injection of glucose (1 g/kg). The values were significantly changed at 0 min (P<0.05), 20 min (P<0.01), and at 50 min (P<0.05). (C) Fold induction of plasma insulin levels one minute after i.v. glucose load. Change is significant, P<0.005. Data are means ±SD; n=10 for both WT and founder A.

20

FIGURE 14

Hypothetical action of *FOXC2* in adipocytes. Filled arrow indicates known positive transcriptional regulation. Open arrow represents a proposed action of *FKHL14*. ADD1/SREBP1 both activates transcription of acetyl CoA carboxylase (Lopez et al.,

25 1996), fatty acid synthase (FAS), and lipoprotein lipase (LPL), and increases the transcriptional activity of PPARγ (Fajas et al., 1999). PPARγ activates transcription of fatty acid transport protein (FATP), acyl-CoA synthetase genes (ACS; Martin et al., 1997), adipocyte fatty acid-binding protein 422/aP2 (aP2; Tontonoz et al., 1994a), LPL (Schoonjans et al., 1996), and phosphoenolpyruvate carboxykinase (PEPCK; Tontonoz

30 et al., 1995). C/EBPα activates insulin-responsive glucose transporter-4 (GLUT4; Kaestner et al., 1990), aP2 (Christy et al., 1989), uncoupling protein 1 (UCP1; Yubero et al., 1994), the insulin receptor (InsR), insulin receptor substrate-1 (IRS-1; Wu et al.,

14

1999b), and PEPCK (Park et al., 1990). A positive feedback loop between C/EBPα and PPARγ have been suggested ('Vu et al., 1999b).

FIGURE 15

5   Reduction in weight gain in (transgenic) tg mice. Analysis were performed on tg-A mice with wt littermates as controls, fed *ad libitum*, mice were approximately 4-6 months of age. There is a reduction in diet induced weight gains in *FOXC2tg* mice, both in females (p<0.02; a) and males (p<0.03; b), as compared with wt mice, values are means ±SEM, n=4 in each group. Mice were on a high fat diet (58.0% on a caloric
10   basis) for seven weeks (see Methods).

FIGURE 16

Metabolic profile. Analysis were performed on tg-A mice with wt littermates as controls, mice were approximately 4-6 months of age and fed *ad libitum*, values are
15   means ±SEM. **a**, Total body lipid content was analysed as total lipid content of carcasses (see Methods), n=4 in each group, p<0.0004. **b**, Serum triglyceride is lowered in *FOXC2tg* mice, n=4 in each group, p<0.004. **c**, Serum cholesterol, no significant difference between wt and tg mice, n=4 in each group. **d**, Plasma free fatty acids (FFA) is lowered in tg mice as compared with wt littermates, n=4 in each group, p<0.02. **e**,
20   Non-fasting plasma glucose is lowered in *FOXC2* tg mice, n=20 in each group, p<0.01. **f**, Plasma insulin is lowered in tg mice, analysis was performed on the same animals as in **e**, p<0.001. There is a reduction in diet induced weight gains in *FOXC2tg* (n=7) mice as compared with wt (n=9; p<0.01; **g**). Whereas the body lipid content after a high fat diet (**h**) increases for both wt and tg mice, as compared with mice on a standard diet (**a**),
25   we note a reduction for tg (n=4) mice as compared with wt (n=6; p<0.0001) in **h**. The weight gain to grams of eaten food ratio was calculated for wt (n=10) and tg (n=9) mice on a control diet (**i**), no significant differences could be detected. In the corresponding experiment, with mice on a high fat diet, there is a 30% reduction (p<0.01) for tg (n=7) as compared wt (n=9) mice (**i**). No gender differences were observed.

30

FIGURE 17

Intravenous glucose tolerance test. Wt and tg-A mice were fed a control (**a**, **b**) or high fat (**c**, **d**) diet for 14 weeks (for details see under "Methods"). After intravenous (i.v.)

15

injection of glucose (1 g/kg), blood samples were drawn immediately before and at 1, 5,
20, 50 and 75min. Plasma glucose levels differ significantly between wt and tg mice
both on a control (a, tg n=8, wt n=9; p<0.02) as well as on a high fat diet (c, tg=5, wt=6;
p<0.001). Plasma insulin values differ also significantly between wt and tg mice both on

5   a control diet (b, p<0.001) and a high fat diet (d, p<0.001). Values are means ±SEM, no
gender differences were observed.

FIGURE 18

Altered β-adrenergic sensitivity, PKA isozyme composition and activation kinetics in

10   adipose tissue from *FOXC2* tg-A mice. (a) Left panel: CAT reporter activity directed
from the RIα promoter transfected together with *FOXC2* expression vector or control
vector into 3T3-L1 pre-adipocytes. Data (n=3 experiments) are CAT values normalised
for transfection efficiency, expressed as fold induction. Right panel: RIα and GAPDH
mRNA levels in WAT from wild type (wt) and the three founder strains (A, B, C) over-

15   expressing *FOXC2*. (b) Levels of RIα and RIIβ immunoreactive protein in WAT and
BAT from transgenic animals (n=4) compared to wild type litter mates (expressed
relative to wild type levels). (c) Kinase activities using Kemptide as substrate in the
presence (total, open bars) or absence (free, solid bars) of 5 μM cAMP. Activities in
transgenic WAT and BAT (n=4) are shown relative to those in wild type litter mates.

20   (d) Kinase activities towards Kemptide measured in WAT homogenates from transgenic
and wild type littermate mice incubated with increasing concentrations of cAMP. Data
are representative of 2 pairs of animals analysed. Total cAMP-inducible activity was set
to 100%. Right panel, immunoblot demonstrating WAT levels of RIα and RIIβ.
Intracellular cAMP levels in adipocyte suspensions prepared from WAT of transgenic

25   animals and wild type littermates (each pooled from 3 animals) and stimulated with
either (e) a non-selective β-agonist (isoproterenol, 1 μM) or (f) a β3-selective agonist
(CGP-12177A,1 μM). Equal amounts of cells were withdrawn at different time points
(0 to 10 min), mixed with stop buffer and flash-frozen. Mean ± half range (SEM) of
duplicate determinations are shown.

30

<center>16</center>

DISCLOSURE OF THE INVENTION

According to the present invention, the human transcription factor gene *FOXC2* is
identified as a key regulator of adipocyte metabolism. Increased *FOXC2* expression, in
5   white (WAT) and brown adipose tissue (BAT), has a pleiotropic effect on gene
expression, which leads to resistance to diet induced weight gain and a decrease in: total
body lipid content, serum triglycerides, plasma levels of free fatty acids, glucose and
insulin. To our knowledge, *FOXC2* is the hitherto only identified gene that, in a
concerted action, can counteract most, if not all, of the symptoms associated with
10 obesity, including hypertriglyceridemia and insulin resistance; a likely consequence
hereof would be protection against type 2 diabetes.

During adulthood, the human winged helix gene *FOXC2* is expressed exclusively in
adipose tissue. The LPL mRNA levels in the *FOXC2* transgenic mice seem to be
15 slightly elevated (Fig. 9), which is in concordance with the initial findings that the two
winged helix cis-regulatory elements are responsible for the inducibility of the LPL
promoter (Enerback *et al.*, 1992). The higher expression level of LPL most probably is
responsible for the significantly decreased plasma TG levels noticed in *FOXC2*
transgenic mice (Fig. 10), in addition to the fact that the profound up-regulation of
20 adipsin in both WAT and BAT (Fig. 9) most certainly is of great importance. Adipsin is
a secreted protein necessary for the formation of acylation stimulating protein (ASP),
which has potent anabolic effects on human adipose tissue for both glucose and free
fatty acid (FFA) storage (Cianflone *et al.*, 1995).

25 The extensive alternations seen in the *FOXC2* transgenic mice presented here predicts a
very central and important role for this winged helix gene hitherto unknown to
participate in molecular events in adipose tissue. It has been demonstrated by gene
targeting experiments that the mouse homologue, *Mfh1*, plays a crucial role during
embryonic development (Iida *et al.*, 1997; Winnier *et al.*, 1997), but so far nothing has
30 been published about its function in adult mice. In a recent publication an *Mfh1/Pax1*
double mutant was shown to be totally absent of BAT at day 15.5 dpc (Furumoto *et al.*,
1999).

17

The *FOXC2* transgenic mice had a clear reduction in white adipose tissue mass. The reduced size of the white fat depots might be due solely to the reduction in size of the adipocytes (Fig. 7), but one cannot rule out the possibility that there is also a reduction in adipocyte number. There are a number of possible reasons why the *FOXC2* transgenic mice have decreased size of white adipocytes. One might presume an increased lipolytic activity of their adipocytes in general; coupled with the greater mass of brown fat this may lead to increased energy expenditure through heat production by BAT giving rise to the lean phenotype. The upregulation of β_3-AR seen in transgenic WAT (Fig. 9) will result in an elevated activation of HSL hence an increase in lipolysis (Fig. 1) ultimately leading to reduced lipid storing.

Furthermore, the transgenic mice were insulin sensitive (Fig. 13); this could be explained by both the upregulation of genes involved in insulin action (InsR, IRS-1, IRS-2, and GLUT4; Fig. 9) and the lean body composition (Fig. 11). Insulin has a critical role in lipid metabolism, promoting the storage of triglycerides in adipocytes through numerous actions on this cell. Among these are stimulation of glucose uptake and inhibition of lipolysis, which occur very rapidly through insulin-responsive glucose transporter protein (GLUT 4) translocation or covalent modification of HSL, respectively. Insulin also stimulates fatty acid and triglyceride synthesis, through the induction of key lipogenic enzymes and induction of lipoprotein lipase. The data presented here are compatible with an increased energy turnover in the adipocytes derived from *FOXC2* transgenic mice.

The white fat depots of *FOXC2* transgenic mice had an ectopic expression of the brown fat specific marker UCP1 (Fig. 9). The origin of multilocular adipocytes in transgenic WAT (Fig. 7d) remains an enigma. It has been suggested that there is a pool of intraconveritble cells or small brown preadipocytes present in WAT. Studies on both rat and mice have demonstrated atypical occurrence of UCP1 in certain WAT depots previously thought to contain only white adipocytes (Cousin *et al.*, 1992; Loncar, 1991). If submitted to cold or to treatment with a β_3-AR selective agonist, UCP1 expression was increased in WAT as in typical BAT and on histological sections one could identify small multilocular cells interspersed between the white adipocytes, which were shown to contain UCP1 by immunhistochemistry (Cousin *et al.*, 1992; Ghorbani *et al.*, 1997).

19

Furthermore, both UCP1 and β$_3$-AR mRNAs have been detected in white fat depots of human beings (Krief *et al.*, 19)3) and recently, it has been shown that cultures of human adipocytes derived from white fat depots express UCP1 after treatment with β$_3$-AR agonists (Champigny and Ricquier, 1996). Moreover, transgenic mice overexpressing

5   β$_1$-AR in WAT and BAT have abundant appearance of brown fat cells in subcutaneous WAT (Soloveva *et al.*, 1997). We would like to speculate that these suggested intraconveritble cells or small brown preadipocytes have undergone proliferation in our transgenic mice, readily detectable on histological sections as small multilocular cells (Fig. 8d), due to the increased expression of β$_3$-AR. The *FOXC2* transgene possibly

10   activates other proteins further down the signal transduction pathway finally leading to the induction of UCP1 expression. In addition, the levels of C/EBPα and PPARγ mRNAs in transgenic WAT reaches the ones in wild-type BAT (Fig. 10) and both of these transcription factors are known to induce UCP1 expression (Digby *et al.*, 1998; Yubero *et al.*, 1994). C/EBPα activates several adipocyte-specific genes and also genes

15   involved in insulin action (Fig. 15), hence C/EBPα (-/-) cells show a complete absence of insulin-stimulated glucose transport, secondary to reduced gene expression and tyrosine phosphorylation for the insulin receptor and IRS-1 (Wu *et al.*, 1999b). The WAT of *FOXC2* transgenic mice have marked elevation of the mRNA levels for both C/EBPα and PPARγ, these transcription factors may in turn be responsible for

20   upregulation of mRNA levels for aP2, LPL, UCP1, GLUT4, insulin receptor, and IRS-1 (Fig. 14)

It is interesting to note that the white adipocytes of *FOXC2* transgenic mice have not just converted into brown adipocytes, in the meaning of mRNA expression, as they have

25   for example higher levels of certain mRNAs (i.e. β$_2$-AR, insulin receptor, IRS-1, and IRS-2) than seen in any type of wild-type adipose tissue.

In the mice studied here, *FOXC2* transgene expression was under the control of the aP2 promoter, which only functions in adipocytes and not in stem cells and probably neither

30   in intraconveritble cells discussed above (Ailhaud *et al.*, 1992). Considering the fact that aP2 is a late marker (Fig. 2) it is quite surprising that we obtain such a dramatic change in the characteristics of white adipocytes. Currently, it is not known if adipocyte dedifferentiation occurs *in vivo*, whereas it has been demonstrated *in vitro* that this

process occurs and is induced by TNFα in human adipocytes (Petruschke and Hauner, 1993). Another interpretation for the occurrence of small multilocular cells in WAT of our transgenic mice could then be a dedifferentiation of originally white adipocytes followed by a conversion into the type of adipocytes observed in the *FOXC2* transgenic mice.

The interscapular brown fat of *FOXC2* transgenic mice weighed ~7.5 times as much as wild-type brown fat (Fig. 8a). This extreme hypertrophy might be explained by the increased expression of β_3- and β_2-AR mRNA seen in BAT of *FOXC2* transgenic mice (Fig. 9). Chronic treatment with β_3-AR agonists increases body temperature and energy expenditure and it causes hypertrophy of the interscapular BAT, with several fold increases in the content of UCP1 and cytochrome oxidase (Himms-Hagen *et al.*, 1994). The morphology of transgenic interscapular BAT is somewhat changed having larger fat droplets than wild-type BAT (Fig. 7a & b). This is not a feature of chronic β_3-AR agonist treatment, but there is a possibility that the upregulation of markers involved in insulin action (Fig. 9) and elevated levels of adipsin promotes the increased storage of triglycerides in brown adipocytes of transgenic mice. It has also been noticed before that elevated levels of UCP2 mRNA can be coupled to this phenotype (Enerback *et al.*, 1997; Kozak *et al.*, 1991).

Dysfunctional BAT seen in the ADD1/nSREBP-1c transgene (Shimomura *et al.*, 1998) and genetically ablated BAT in the UCP1-DTA transgene (UCP1 promoter – diphtheria toxin A chain) (Lowell *et al.*, 1993) leads to insulin resistance. Transgenic mice overexpressing ADD1/nSREBP-1c displays several features quiet opposite the ones of the *FOXC2* transgene, including insulin resistance and NIDDM. *FOXC2* transgenic mice displays a somewhat opposite change of expression pattern compared with that of ADD1/nSREBP-1c transgenic mice, there the mRNAs encoding PPARγ, C/EBPα, aP2, UCP1, adipsin, InsR, IRS-1, IRS-2, and GLUT4 all are downregulated. In our transgene all of this mRNAs are instead upregulated (Fig. 9). However, intriguingly our transgenic mice actually have raised levels of ADD1/SREBP1 mRNA in WAT (Fig. 9) somewhat mimicking the ADD1/nSREBP-1c transgene in that regard.

20

FOXC2 might be an important participant in the regulation of leptin expression, based on the fact that leptin mRNA levels are down-regulated in *FOXC2* transgenic mice (most prominent in BAT; Fig. 9), and the ten-fold decrease of Ob promoter activity seen in cell culture experiments then cotransfected with *FOXC2* expression plasmid (Fig. 4).

5   In addition, leptin expression is inhibited by β_3-adrenergic stimuli (Mantzoros *et al.*, 1996), which is presumed to be high in *FOXC2* transgenic mice due to the up-regulation of β_3-AR mRNA levels.

The amount of food consumed by transgenic mice compared to that of wild-type did not
10   differ (Fig. 8d), and no significant difference in body weight has been observed, predicting that the difference observed in total body lipid content must be compensated with an increased anabolism in *FOXC2* transgenic mice. The *FOXC2* transgenic mice most probably also are protected against developing diet-induced obesity, taking in consideration the observed insulin sensitivity (Fig. 13), the lower blood glucose levels
15   and more efficient glucose elimination (Fig. 12) observed in our transgenic mice. Insulin sensitivity and/or resistance to diet-induced obesity have been observed for several other transgenic mouse models: targeted disruption of the RIIβ subunit of protein kinase A results in lean mice resistant to diet-induced obesity (Cummings *et al.*, 1996), mice lacking the protein tyrosine phosphatase-1B gene (PTP-1B) are insulin
20   sensitive and resistant to obesity (Elchebly *et al.*, 1999), aP2-UCP1 transgenic mice are prevented against genetic obesity (Kopecky *et al.*, 1995), and transgenic mice overexpressing the β_1-AR in adipose tissue are resistant to obesity (Soloveva *et al.*, 1997). Moreover, β_3-AR agonists have been found to have anti-diabetic effects in animal models of obesity and NIDDM; chronic dosing can improve glucose tolerance,
25   increase insulin sensitivity and reduce fasting blood glucose levels (Cawthorne *et al.*, 1992). *FOXC2* is the only adipocyte specific gene that, directly or indirectly, regulates triglyceride metabolism, adrenergic regulation and insulin action in adipocytes. Actually, the *FKHL14*, is to our knowledge the only known gene that, in a concerted action, can counteract most, if not all, of the symptoms associated with obesity:
30   hypertriglyceridemia, insulin resistance and most likely the associated clinical syndrome of NIDDM.

21

The apparent *FOXC2* transgene dose responsive effect observed in WAT for the induction of UCP1, β_3-AR, and adipsin, may indicate a direct interaction for *FOXC2* with the promoters of these genes. A schematic view of the hypothetical action of *FOXC2* in adipocytes is shown in Fig. 14.

According to the present invention, proper activation of *FOXC2* by drugs may decrease fat stores, while preserving skeletal muscle mass, by preventing fat assimilation during digestion and by increasing WAT lipolysis, BAT thermogenesis, and insulin action. Such drugs may thus prove useful in treating obesity and NIDDM as well as associated diseases. It is thus foreseen that an effective amount of a polypeptide encoded by the human *FOXC2* gene, could be useful in methods for the treatment of medical conditions related to obesity.

In another aspect, this invention relates to a construct, or more specifically a gene construct or recombinant construct, comprising a human *FOXC2* nucleotide sequence operably linked to an element selected from the group consisting of promoters, response elements, enhancer elements and mixtures thereof. The term "operably linked" as used herein means functionally fusing an element with a structural gene in the proper frame to express the structural gene under control of the element.

Preferably, the said element is a promoter, in particular an adipose-specific promoter such as the adipose-specific promoter of the murine gene encoding adipocyte P2 (Fig. 4), which can be isolated as described by Ross et al. (1990).

In a preferred form of the invention, the said *FOXC2* nucleotide sequence is identical or substantially similar with SEQ ID NO: 1 of the Sequence Listing. However, the *FOXC2* nucleotide sequence is not to be limited strictly to the sequence shown as SEQ ID NO: 1. Rather the invention encompasses constructs comprising nucleotide sequences carrying modifications like substitutions, small deletions, insertions or inversions, which nevertheless encode polypeptides having substantially the biochemical activity of the FOXC2 polypeptide.

Consequently, included in the invention are constructs wherein the said human *FOXC2* nucleotide sequence is selected from:

(a) the nucleotide sequence shown as SEQ ID NO: 1;

(b) nucleotide sequences capable of hybridizing, under stringent hybridization

5   conditions, to a nucleotide sequence complementary to the polypeptide coding region of a nucleotide sequence as defined in (a) and which codes for a biologically active FOXC2 polypeptide, or a functionally equivalent modified form thereof;

(c) nucleic acid sequence which are degenerate as a result of the genetic code to a nucleotide sequence as defined in (a) or (b) and which codes for a biologically active

10   FOXC2 polypeptide, or a functionally equivalent modified form thereof; and

(d) nucleotide sequences which are at least 90% homologous, preferably at least 95% homologous, with the nucleotide sequence shown as SEQ ID NO: 1 in the Sequence Listing.

15   The term "stringent hybridization conditions" is known in the art from standard protocols (e.g. Ausubel et al) and could be understood as e.g. hybridization to filter-bound DNA in 0.5 M $NaHPO_4$, 7% sodium dodecyl sulfate (SDS), 1 mM EDTA at +65°C, and washing in 0.1xSSC / 0.1% SDS at +68°C.

20   In another aspect, the invention provides a transgenic non-human mammalian animal whose genome comprises a gene construct as defined above, said animal being capable of expressing the human *FOXC2* gene in its adipose tissue.

By "transgenic animal" is meant a non-human mammalian animal that includes a

25   nucleic acid sequence which is inserted into a cell and becomes a part of the genome of the animal that develops from that cell. Such a transgene may be partly or entirely heterologous to the transgenic animal. Although transgenic mice represent a preferred embodiment of the invention, other transgenic mammals, including transgenic rodents (for example, hamsters, guinea pigs, rabbits, and rats), and transgenic pigs, cattle, sheep,

30   and goats may be constructed by standard techniques and are included in the invention.

One means available for producing a transgenic animal, with a mouse as an example, is as follows: Female mice are mated, and the resulting fertilized eggs are dissected out of

23

their oviducts. The eggs are stored in an appropriate medium. DNA or cDNA encoding the *FOXC2* gene is purified from a vector by methods well known in the art. Tissue specific regulatory elements, such as the adipocyte-specific aP2 promoter discussed above, may be fused with the coding region to permit tissue-specific expression of the

5   trans-gene. The DNA, in an appropriately buffered solution, is put into a microinjection needle and the egg to be injected is put in a depression slide. The needle is inserted into the pronucleus of the egg, and the DNA solution is injected. The injected egg is then transferred into the oviduct of a pseudopregnant mouse, where it proceeds to the uterus, implants, and develops to term.

10

A transgenic mouse according to the invention could preferably be derived from a genetically obese mouse. Genetically obese mice, such as ob/ob or db/db mice, are well known in the art.

15   In a further aspect, the invention provides an isolated cell line derived from the transgenic non-human mammalian animal. In yet another aspect, the invention provides a method for producing a transgenic non-human mammalian animal overexpressing the human *FOXC2* gene, said method comprising chromosomally incorporating a gene construct comprising the human *FOXC2* gene, together with suitable regulatory

20   sequences, into the genome of said non-human mammalian animal.

The invention also provides a method for studying the biological activity of a polypeptide encoded by the human *FOXC2* gene, said method comprising the steps (i) producing a transgenic non-human mammalian animal overexpressing the human

25   *FOXC2* gene; and (ii) comparing the phenotype of the said transgenic non-human mammalian animal with a wild-type animal of the same species.

In further important aspects, the invention provides biological screening assays for the identification of compounds that could be useful for the treatment of medical conditions

30   related to obesity, or alternatively, to malnutrition. A "medical condition related to obesity" includes e.g. obesity, NIDDM, hypertension and hyperlipidemia. The said "medical condition related to malnutrition" includes e.g. anorexia, ineffective metabolism, and cancer.

24

Consequently, the invention provides a method for identifying a compound useful for the treatment of a medical condition related to obesity, said method comprising the steps (i) contacting a test compound with the human *FOXC2* gene; and (ii) determining whether said test compound activates the expression of the human *FOXC2* gene, such

5   activation being indicative for a compound useful for the treatment of a medical condition related to obesity.

In another aspect, the invention provides a method of screening for a compound useful for the treatment of a medical condition related to obesity, said method comprising

10   exposing a non-human mammalian animal to a test compound, and determining the activity of said human *FOXC2* gene in said non-human mammalian animal, wherein an increase in said gene activity as compared to an untreated non-human mammalian animal being indicative of a compound useful for the treatment of a medical condition related to obesity.

15

The said non-human mammalian animal is preferably a mouse, for example an obese mouse. Mice can be rendered obese by administration of a high-fat diet. Alternatively, the mouse can be a genetically obese mouse, such as an ob/ob or db/db mouse.

20   In the methods described above, the activity of the human *FOXC2* gene in said non-human mammalian animal, can advantageously be compared to the activity of the said human *FOXC2* gene in a transgenic non-human mammalian animal expressing or overexpressing the said human *FOXC2* gene.

25   In an alternative method for identifying a compound useful for the treatment of a medical condition related to obesity, the method comprises the steps (i) contacting a test compound with a polypeptide encoded by the human *FOXC2* gene; and (ii) determining whether said test compound stimulates the biological activities of the said polypeptide, such stimulation being indicative for a compound useful for the treatment of a medical

30   condition related to obesity. The term "biological activities", as used in this context, means e.g. enhancing the DNA-protein interaction between the FOXC2 polypeptide and target sequences in promoters of target genes.

2$\mathcal{S}$

The invention further provides a method for identifying a compound useful for the treatment of a medical condition related to malnutrition, said method comprising the steps (i) contacting a test compound with the human *FOXC2* gene; and (ii) determining whether said test compound decreases or inhibits expression of the *FOXC2* gene, such decrease or inhibition being indicative for a compound useful for the treatment of a medical condition related to malnutrition.

Also included in the invention is a method of screening for a compound useful for the treatment of a medical condition related to malnutrition, said method comprising exposing a non-human mammalian animal, preferably a mouse, such as an obese mouse, to a test compound, and determining the activity of said human *FOXC2* gene in said non-human mammalian animal, wherein a decrease in said gene activity as compared to an untreated non-human mammalian animal being indicative of a compound useful for the treatment of a medical condition related to malnutrition.

In an alternative method for identifying a compound useful for the treatment of a medical condition related to malnutrition, the method comprises the steps (i) contacting a test compound with a polypeptide encoded by the human *FOXC2* gene; and (ii) determining whether said test compound decreases or inhibits the biological activities of the said polypeptide, such decrease or inhibition being indicative for a compound useful for the treatment of a medical condition related to malnutrition.

In the above discussed methods for identifying compounds decreasing or stimulating *FOXC2* biological activity, other genes encoding forkhead proteins can conveniently be used for comparison (counter-screening) in order to determine the specificity of the test compound. Such forkhead genes, e.g. freac-3 and freac-4, are disclosed e.g. by Pierrou et al. (1994) EMBO Journal 13, 5002-5012.

The skilled person will appreciate that other polypeptides, interacting in the same biological pathways as the FOXC2 polypeptide, will be useful in screening methods for the identification of agents, which agents are useful for the treatment of medical conditions related to obesity or malnutrition. Such alternative target polypeptides could be identified e.g. by a yeast two-hybrid assay (cf. Example 11), or by microarray technology (cf. Example 14). Consequently, the invention includes a method for

identifying an agent useful for the treatment of a medical condition related to obesity, said method comprising the steps:

(i) identifying a target polypeptide interacting with the FOXC2 polypeptide;

(ii) contacting a candidate agent with the said target polypeptide; and

5   (iii) determining whether said candidate agent stimulates the biological activities of the FOXC2 polypeptide, such stimulation being indicative for a compound useful for the treatment of a medical condition related to obesity.

The phrase "determining whether said candidate agent stimulates the biological

10   activities of the FOXC2 polypeptide" is intended to comprise, for instance, determining whether the candidate agent is capable of increasing expression of the FOXC2 polypeptide, or whether the candidate agent is capable of otherwise modulating the biological activities of the FOXC2 polypeptide. The said biological activities of the FOXC2 polypeptide are discussed in detail in the Examples below, and comprises e.g. a

15   reduction of total lipid content in test animals.

For therapeutic uses, the compositions or agents identified using the methods disclosed herein may be administered systemically, for example, formulated in a pharmaceutically acceptable buffer such as physiological saline. Preferable routes of administration

20   include, for example, oral, subcutaneous, intravenous, intraperitoneally, intramuscular, or intradermal injections, which provide continuous, sustained levels of the drug in the patient. Treatment of human patients or other animals will be carried out using a therapeutically effective amount of an identified compound in a physiologically acceptable carrier. Suitable carriers and their formulation are described, for example, in

25   Remington's Pharmaceutical Sciences by E. W. Martin. The amount of the active compound to be administered varies depending upon the manner of administration, the age and body weight of the patient, and with the type of disease and extensiveness of the disease. Generally, amounts will be in the range of those used for other agents used in the treatment of obesity and diabetes.

30

In a further important aspect of the invention, the human *FOXC2* gene could be used in gene therapy of medical conditions related to obesity. Included in the invention is thus a method of treating obesity in a human, comprising (i) administering to the said human a vector comprising a human *FOXC2* DNA sequence operably linked to a promoter; and

27

(ii) allowing the said human to express a therapeutically effective amount of a polypeptide encoded by said human *FOXC2* gene. A gene delivery system including said vector, comprising a human *FOXC2* DNA sequence operably linked to a promoter, is in itself another aspect of the invention.

5

The *FOXC2* gene should be operably linked to at least one element which allows for expression of the gene when introduced into the host cell environment. These sequences include promoters, response elements, and enhancer elements. Preferred is the adipose-specific promoter/enhancer of the murine gene encoding adipocyte P2

10

The heterologous gene may be delivered to the organism using a vector or other delivery vehicle. DNA delivery vehicles can include viral vectors such as adenoviruses, adeno-associated viruses, and retroviral vectors. See, for example: Chu et al. (1994) Gene Ther 1: 292-299; Couture et al. (1994) Hum Gene Ther 5:667-677; and Eiverhand et al. (1995) Gene Ther 2: 336-343. Non-viral vectors which are also suitable include DNA-lipid complexes, for example liposome-mediated or ligand/poly-L-Lysine conjugates, such as asialoglyco-protein-mediated delivery systems. See, for example: Feigner et al. (1994) J. Biol. Chem, 269: 2550-2561; Derossi et al. (1995) Restor. Neurol. Neuros. 8: 7-10; and Abcallah et al. (1995) Biol. Cell 85: 1-7.

20

If a vector is chosen as the delivery vehicle for the gene, it may be any vector which allows expression of the gene in the host cells. It is preferable if the vector also is one that is capable of integrating into the host genome, so that the gene can be expressed permanently. Ad (adenovirus) vectors have been exploited for the delivery of foreign genes to cells for a number of reasons, including the fact that Ad vectors have been shown to be highly effective for the transfer of genes into a wide variety of tissues *in vivo* and the fact that Ad infects both dividing and non-dividing cells. The vector is administered to the host, generally by intravenous injection. Suitable titers will depend on a number of factors, such as the particular vector chosen, the host, strength of promoter used and the severity of the disease being treated.

Alternatively, it is contemplated that in some human disease states, preventing the expression of, or decreasing the activity of, the human FOXC2 gene will be useful in treating disease states. It is contemplated that antisense therapy or gene therapy could be

2ρ

applied to negatively regulate the expression of the human FOXC2 gene. Antisense

nucleic acids (preferably 10 to 20 base-pair oligonucleotides) capable of specifically

binding to FOXC2 expression control sequences or FOXC2 RNA are introduced into

cells (e.g. by a viral vector or colloidal dispersion system such as a liposome). The

5   antisense nucleic acid binds to the FOXC2 target nucleotide sequence in the cell and

prevents transcription and/or translation of the target sequence. Phosphorothioate and

methylphosphonate antisense oligonucleotides are specifically contemplated for

therapeutic use by the invention. The antisense oligonucleotides may be further

modified by poly-L-lysine, transferrin polylysine, or cholesterol moieties at their 5'-end.

10   Suppression of FOXC2 expression at either the transcriptional or translational level is

useful to generate cellular or animal models for diseases/conditions characterized by

aberrant FOXC2 expression.

Throughout this description the terms "standard protocols" and "standard procedures",

15   when used in the context of molecular biology techniques, are to be understood as

protocols and procedures found in an ordinary laboratory manual such as: Current

Protocols in Molecular Biology, editors F. Ausubel et al., John Wiley and Sons, Inc.

1994, or Sambrook, J., Fritsch, E.F. and Maniatis, T., Molecular Cloning: A laboratory

manual, 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY 1989.

20

EXPERIMENTAL METHODS

Cloning and DNA construct

25

A human adipose tissue λgt11 cDNA library (Clontech) was screened with a probe

mixture corresponding to the conserved fork-head domain derived from: FOXC1,

FOXD1, FOXL1, and FOXA1. Hybridization was carried out at low stringency, *i.e.*

6xSSC at +60°C, post-hybridization washes at 0.5xSSC at +60°C. One of the positive

30   recombinants harboring a 2.1 kb insert was subcloned and sequenced. A 5.4-kb *Eco*RV-

*Sma*I fragment was excised from pBluescript II SK(+) vector containing the 5.4-kb

promoter/enhancer of the mouse aP2 gene and ligated into the *Eco*RV-*Spe*I blunt site of

pBluescript II SK(+) vector containing the 2.1 kb FOXC2 cDNA. A 7.6 kb *Xho*I blunt

29

fragment containing the aP2 promoter/enhancer followed by the FOXC2 cDNA was excised from the above plasmid and ligated into the *EcoR*V site of the pCB6+ vector, which contains a polyadenylation signal from the human growth hormone gene. After these procedures the resulting 8.2-kb fragment, harboring the aP2-FOXC2 construct

5   with polyadenylation signal, was flanked by the unique sites *Not*I and *Age*I. The plasmid was sequenced over ligation sites.

Transgenic mice

10   Construct DNA (aP2-FOXC2), purified using Qiagen kit, according to manufacturer's instructions, was injected into the male pronucleus of (C57BL6 x CBA) F$_1$ zygotes, cultured over night and transferred to pseudopregnant females. Tg founder lines were back-crossed to C57BL6/J for four generations. Mice were feed a standard chow with 4% fat content. In experiments with high fat diet mice were fed either a chow with 58%

15   fat or a control diet with 11.4% fat (on a caloric basis; Research Diets) for 7 weeks. High fat chow has a total energy content of 23.4KJ/g, control diet 12.6KJ/g.

Histology

20   Tissues were fixed over night in 4% paraformaldehyde in PBS at +4°C, dehydrated, embedded in paraffin, sectioned (6-8 μm) and stained with haematoxylin and eosin.

Serum and lipid analysis

25   Plasma insulin was determined radioimmunochemically with the use of a guinea pig anti-rat insulin antibody, ^{125}I-labeled porcine insulin as tracer and rat insulin as standard (Linco). Free and bound radioactivity was separated by use of an anti-IgG (goat anti-guinea pig) antibody (Linco). The sensitivity of the assay is 17 pmol/l and the coefficiency of variation is less than 3% at both low and high levels. Plasma glucose

30   was determined with the glucose oxidase method and FFA was measured photometrically. Plasma glucagon was determined radioimmunochemically with the use of a guinea pig antiglucagon antibody specific for pancreatic glucagon, ^{125}I-labelled-glucagon as tracer, and glucagon standard (Linco). Free and bound radioactivity was

separated by use of an anti-IgG (goat anti-guinea pig) antibody (Linco). The sensitivity of the assay is 7.5 pg/ml and t ie coefficient of variation is less than 9%. Blood levels of serum cholesterol and triglycerides were determined by fully enzymatic techniques [39,40]. Total body lipid was assessed using alcoholic hydroxide digestion with

5   saponification of all lipids, neutralization, followed by enzymatic determination of glycerol.

Intravenous glucose tolerance test

10   The mice were anesthetized with an intraperitoneal injection of midazolam 0.4 mg/mouse (Hoffman-La-Roche) and a combination of fluanison (0.9 mg/mouse) and fentanyl 0.02 mg/mouse (Janssen). Thereafter, a blood sample was taken from the retrobulbar, intraorbital, capillary plexus in heparinized tubes, and D-glucose 1g/kg (British Drug Houses) was injected rapidly intravenously. New blood samples were

15   taken after 1, 5, 20, and 50 minutes. Following immediate centrifugation at +4°C, plasma was separated and stored at −20°C or until analysis.

Northern blot

20   cDNA probes for mouse FoxC2, aP2, ADD-1/SREBP1, coxII, adipsin, β_{1-3}-AR, GLUT4, IR, IRS1, and IRS2 were prepared by RT-PCR by use of first-strand cDNA from mouse epididymal fat poly(A)$^+$ RNA. The PCR primers used to generate these probes were as follows:

25   FoxC2: 5' primer, GCTTCGCCTCCTCCATGGGAA and 3' primer, GGT TACAAATCCGCACTCGTT (GenBank # Y08222).

aP2: 5' primer, CTC CTG TGCTGCAGCCTTTCTC and 3' primer, CGTAACTCACCACCACCAGCTTGTC (GenBank # M13261).

30

ADD1/SREBP-1: 5' primer, GCCAACTCTCCTGAGAGCTT and 3' primer, CTCCTGCTTGAGCTTCTGGTT (GenBank # AB017337).

31

CoxII: 5' primer, CCATTCCAACTTGGTCTACAA and 3' primer, GGAACCATTTCTAGGACAATG (GenBank # J01420).

Adipsin: 5' primer, CGAGGCCGGATTCTGGGTGGCCAG and 3' primer,
5   TCGATCCACATCCGGTAGGATG (GenBank # X04673).

β_1-AR: 5' primer, CGGCTGCAGACGCTCACCAA and 3' primer, CGCCACCAGTGCATGAGGAT (GenBank # L10084).

10   β_2-AR: 5' primer, GCTGCAGAAGATAGACAAAT and 3' primer, GGGATCCTCACACAGCAGTT (GenBank # X15643).

β_3-AR: 5' primer, CTGCTAGCATCGAGACCTT and 3' primer, CGAGCATAGACGAAGAGCAT (GenBank # X60438).

15

GLUT4: 5' primer,CTCAGCAGCGAGTGACTGGGAC and 3' primer, CCCTGAGTAGGCGCCAATGAGG (GenBank # D28561).

IR: 5' primer, GTAGCCTGATCATCAACATCCG and 3' primer,
20   CCTGCCCATCAAACTCTGTCAC (GenBank # J05149).

IRS1: 5' primer, ATGGCGAGCCCTCCGGATACCG and 3' primer, CCTCTCCAACGCCAGAAGCTGCC (GenBank # X69722).

25   IRS2: 5' primer, GGATAATGGTGACTATACCGAGA and 3' primer, CTCACATCGATGGCGATATAGTT (GenBank # AF090738).

cDNA probes were radiolabeled with [α-^{32}P]dCTP (3000 Ci/mmole) by the random labeling method. Total RNA from mice in each group was pooled, and aliquots of 12 μg
30   were separated on an agarose gel. The filters were hybridized with ^{32}P-labeled probe (10^6 cpm/ml) for 1 h at 62°C with QuikHyb solution (Stratagene) and washed with 0.1% SDS/0.1x SSC at +62°C for 3x20 min.

Transfections and reporter gene analysis

Non-confluent cultures of 3T3-L1 adipocytes were transfected with a CAT reporter (pCAT) driven by the human RIα proximal promoters upstream of the alternatively
5   spliced 1a and 1b leader exons (nucleotides 1509 to 2470 GenBank # Y07641). To control transfection efficiency a pGL3control (Promega) luciferase-encoding vector was used. In cotransfections a *FOXC2* expression vector or vector void of insert was used. Transfections were carried out using lipofectamine (Gibco), followed by CAT and luciferase assays.
10

PKA immunoblotting and kinase activity

WAT and BAT were treated by a Polytron tissue homogenizer (3 x 15 s) and sonicated, on ice, in a buffer containing 10 mM potassium phosphate, pH 6.8, 150 mM sodium
15   chloride, 1 mM EDTA, 10 mM CHAPS and protease inhibitors, and centrifuged (15,000 x g) to remove insoluble material. Protein concentrations were determined by Bradford assays (BioRad). For immunoblotting, 30 µg of protein was separated by 10% SDS-PAGE, transferred to PVDF membranes and incubated with anti-RIα and anti-RIIβ mAb. Primary antibodies were detected by HRP-conjugated anti-mouse IgG
20   (Transduction Laboratories, 1:5000) and ECL (Amersham). PKA activity was measured using Kemptide (Leu-Arg-Arg-Ala-Ser-Leu-Gly) as substrate in the absence or presence of varying concentrations of cAMP. The low levels of activity not inhibited by PKI (2 µM) were subtracted to determine PKA-specific activity.

25

EXAMPLES OF THE INVENTION

Additional features of the invention will be apparent from the following Examples. Examples 1 to 10 are actual, while the remaining Examples are prophetic.

30

33

EXAMPLE 1: Isolation of *FOXC2* from human abdominal fat tissue

To identify presumptive winged helix genes that are expressed in adipose tissue we
screened a Human Fat Cell 5′-STRETCH plus cDNA Library (Clontech) using a low

5   stringency hybridization strategy with a mix of cDNA probes corresponding to DNA-
binding domains from different winged helix proteins, to cover the variation between
different family members. By this technique, we were able to identify three different
forkhead genes, termed *FKHL5*, *FKHL14*, and *FKHL18*. *FKHL5* was known to be
expressed exclusively in lung and placenta (Pierrou *et al.*, 1994) and expression in

10   adipose tissue could not be detected as judged by Northern blot analysis (data not
shown). *FKHL18* was identified as a novel member of the forkhead family (Cederberg
et al., 1997) having a very high homology with the forkhead motif of the mouse gene
fkh-3 (Kaestner *et al.*, 1993). Sequence from outside the forkhead motif have not yet
been published for *fkh-3* but with regard to the discrepancy of the expression patterns,

15   with *FKHL18* being expressed in arterial vessel wall (aorta) and to a lower extent in
kidney and *fkh-3* being expressed in a wide variety of tissues, they are presumed to
constitute two different genes. We were not able to detect any expression of *FKHL18* in
adipose tissue (data not shown). The third identified gene, *FKHL14* (hereinafter referred
to as *FOXC2*; Miura, N. et al. (1997) Genomics 41, 489-492), was shown to be

20   expressed exclusively in human adult adipose tissue by Northern blot analysis (Fig. 3a).
The mouse homologue of *FOXC2* is known as *Mfh1* and is expressed in dynamic
patterns in the paraxial mesoderm of the trunk and head, in the mesenchyme and
endothelial cells of the branchial arches, and in many other sites in the embryo
(Kaestner *et al.*, 1996; Miura *et al.*, 1993; Winnier *et al.*, 1997). It has been

25   demonstrated by gene targeting experiments that *Mfh1* plays a crucial role during
embryonic development. Homozygous null mutants died pre- and perinatally with
multiple skeletal and cardiovascular defects, including defects in the neurocranium and
vertebral column, interruptions or coarctations of the aortic arch, and ventricular septal
defects (Iida *et al.*, 1997; Winnier *et al.*, 1997). Heterozygous mice were

30   indistinguishable from wild-type and appeared healthy. The expression of *Mfh1* in adult
tissues was restricted to WAT and BAT (Fig. 3b).

3 4

EXAMPLE 2: Physiological studies of *FOXC2*

In a Northern blot experiment we used RNA from collagenase prepared adipocytes and cells with lower triglyceride content, mostly preadipocytes/adipocytes and cells of

5   stroma/vascular origin, in order to identify the cell type expressing *FoxC2* (Fig. 3c). As can be deduced from Fig. 3c, *FoxC2* expression is highest in the adipocyte fraction with a lower level of expression in stroma/vascular cells, most likely due to the presence of adipocytes with lower triglyceride content – since the adipocyte specific marker LPL (lipoprotein lipase) also is expressed by these cells.

10

To address the general question of the physiological role/regulation of *FoxC2* we preformed the following experiment: 3T3-L1 adipocytes were stimulated separately and in combination with the cAMP-inducing agent forskolin, the protein kinase C-activator TPA and Ca^{2+}-ionophore A23187 to mimic signals elicited down-stream of G-protein

15   coupled receptors. Whereas forskolin alone or in combination with Ca^{2+}-ionophore produce a 3-fold increase in steady state levels of *FoxC2* mRNA (Fig. 3d), Ca^{2+}-ionophore by itself does not appear to regulate *FoxC2* levels. In contrast, TPA and combinations that include TPA give a strong increase (9 to 10-fold) in *FoxC2* mRNA (Fig. 3d). This suggests that *FoxC2* is regulated by intracellular events similar to those

20   elicited by G-coupled hormone receptors.

EXAMPLE 3: Generation of transgenic mice overexpressing *FKHL14*

25   To elucidate whatever function *FOXC2* might have *in vivo* we decided to create transgenic mice overexpressing *FOXC2* in adipose tissue. To achieve overexpression of *FOXC2* in both white and brown adipose tissue, we prepared a transgene construct (Fig. 4a) encoding *FOXC2* driven by a 5.4-kb DNA fragment containing the adipose-specific enhancer/promoter of the gene encoding adipocyte P2 (Ross *et al.*, 1990). Integration

30   positive mice were identified using Southern blot analysis (Fig. 4b).

We studied three lines of transgenic mice (A, B, and C) that were derived from independent founders. Transgene expression, analyzed by Northern blot using a *FOXC2* (human specific) probe, was detected at high levels in both WAT and BAT for all three

3*5*

founders. The three different founders had a slight difference in expression levels of the
transgene in white fat, founder A having the highest and founder B the lowest, while the
expression levels in brown fat were equal (Fig. 5c). All of the transgenic mice used for
the study were hemizygous for the transgene and from the second to fourth generation.
5   The transgenic mice appeared normal at external inspection and the transgenic allele
was propagated with the expected mendelian distribution. Fed a standard rodent chow
diet (4% fat, 18.5% protein, and 55.7% sucrose) weight gain was studied for all
founders from 4 weeks up to 6 months of age with no significant difference between
nontransgenic and transgenic littermates noticed (data not shown).

10

We selected three independent founder lines, named tg-A, tg-B and tg-C with tg-A
having the highest, tg-B the lowest and tg-C an intermediate level of *FOXC2* expression
in WAT. There is a clear dose-response relation between the expression level of the
transgene and reduction in the relative weight of the intra-abdominal WAT depot (Fig.
15   5a). A similar dose-response pattern exists when the weight ratios of intra-abdominal
WAT to interscapular BAT are compared (Fig. 5b). The relative weight of the
interscapular BAT depot is increased in tg animals but does not exhibit any dose-
response association (Fig. 5a), which is in accordance with the fact that there is no
difference between tg-A, tg-B and tg-C in expression of the transgene in BAT.

20

EXAMPLE 4: WAT of *FOXC2* transgenic mice morphologically resembles BAT

The interscapular brown adipose tissue depot and the intrabdominal white adipose tissue
25   were examined in 5-months-old mice (Fig. 6). Interscapulary, transgenic mice had a
greatly enlarged bilobed (dissected apart in the picture) fat pad with the "looks" of
brown adipose tissue without the usual coverage of white adipose tissue (Fig. 6b),
which normally have to be dissected to uncover the interscapular BAT depot (Fig. 6a).
An exposed abdominal view of *FOXC2* transgenic mice revealed an obvious decrease
30   of intrabdominal fat mass and also a distinct change in the appearance towards the
brown adipose tissue phenotype (Fig. 6d), when compared to the large, pale lipid-
storing intrabdominal fat pad of wild-type mice (Fig. 6c). The adipose tissue change in
appearance and mass between transgenic and non-transgenic mice was very striking
then the depots had been dissected out (Fig. 6e & f).

36

Histological analysis of BAT and WAT from 5-months-old mice revealed profound
differences between transgenic and non-transgenic tissues. The majority of adipocytes
in brown fat of the transgenic mice contained few markedly enlarged fat droplets (Fig.

5   7b), instead of small multilocular fat droplets typical for BAT (Fig. 7a). Adipocytes in
intrabdominal white fat of the transgenic mouse showed heterogeneity of size, all of
them having a clearly reduced size (Fig. 7d). This was in contrast to WAT from wild-
type mouse, which consisted of large adipocytes of uniform size filled with a large,
unilocular lipid-storing vacuole (Fig. 7c).

10

In a cold adaptation experiment (4°C, 4 hours) tg mice display a clear reduction in size ·
and number of triglyceride droplets in BAT (Fig. 7 e–h). This indicates that the changes
in gene expression induced by the transgene will allow a net accumulation of
triglyceride at room temperature (22°C) whereas these triglycerides are metabolised at

15   4°C. In a recent report it has been suggested that, apart from WAT and BAT, aP2 is also
expressed in activated macrophages. Measurements of two cytokines IL-12 and IL-18,
both implicated in macrophage activation, showed no difference between tg and wt
mice (data not shown).

20   To further examine the change of size of fat depots in transgenic mice, we compared the
weights of intrabdominal WAT depots and interscapular BAT depots of 5-months-old
wild-type females (n=3) and *FOXC2* transgenic littermates (n=3). In transgenic mice
there was a decrease in weight of the intrabdominal WAT depot (~40%) whereas the
interscapular BAT depot showed a marked increase (~7.5 times; Fig. 8a). In

25   consequence the ratio between weights of the intrabdominal fat depot and the
interscapular brown fat depot was greatly decreased (Fig. 8b). No significant difference
in body weight could be detected between transgenic and non-transgenic mice (Fig. 8c),
nor could any difference in food consumption be observed then measured during a time
period of two months (Fig. 8d).

30

37

EXAMPLE 5: Altered gene expression in *FOXC2* transgenic mice

Figure 9 shows an analysis of mRNA steady state levels in white and brown adipose tissue of wild-type mice and the three independent transgenic founders. In wild-type
5   mice, the mRNA for uncoupling protein 1 (UCP1) was detectable only in BAT, but in the transgenic mice expression could be detected in WAT, in a dose responsive manner in proportion to the expression level of transgenic *FKHL14*. The WAT blot and BAT blot had different exposure times, 2 days and 30 minutes respectively. UCP2 seemed to have a tendency to be regulated in the opposite direction as compared with UCP1 in
10 WAT of transgenic mice. The uncoupling protein expressed in skeletal muscle and BAT, UCP3, showed greatly decreased levels in transgenic BAT. The cytochrome c-oxidase subunit II (CoxII), a gene encoded by the mitochondrial genome, was used as a marker for density of mitochondria. The density of mitochondria in WAT and BAT from transgenic animals appeared to be elevated and to a lesser extent reduced,
15 respectively. In wild-type mice, the mRNAs of β_1- and β_3-AR were much lower in WAT than in BAT. The *FOXC2* transgene abolished this discrepancy, raising these mRNAs selectively in WAT so that they became equal, or in the case of β_3-AR even higher, compared to the levels in BAT. The β_2-AR mRNA level was elevated in both WAT and BAT of transgenic mice, reaching levels not seen in nor WAT or BAT of
20 wild-type littermates. White fat depots of transgenic animals exhibited profound increment in four of its mRNAs that are associated with fully differentiated adipocytes, that is, PPARγ2, C/EBPα, aP2, and adipsin. Furthermore PGC-1, a co-activator of PPARγ2, was upregulated in both WAT and BAT. The mRNA for ADD1/SREBP1 was elevated in WAT for all three founders. The transgenic animals demonstrated a
25 reduction of the amount of leptin mRNA with the most distinct effect in BAT. Steady state levels of LPL mRNA appear to have increased slightly in WAT of transgenic mice. All investigated markers involved in insulin action were upregulated: the insulin receptor (InsR), IRS-1, IRS-2, and insulin-responsive glucose transporter-4 (GLUT4). The upregulation was most evident in WAT. GAPDH was used as a control to verify
30 equal amounts of total RNA in the different lanes.

3ρ

EXAMPLE 6: Triglyceride co ntent is altered in *FOXC2* transgenic mice

Serum triglyceride content wa.; analyzed for 14-weeks-old wild-type males (n=4) and *FOXC2* transgenic littermates (n=4) fed ad libitum. The transgenic animals exhibited a

5   ~60% reduction in serum triglyceride levels (Fig. 10).

Total body lipid content was assessed using alcoholic potassium hydroxide digestion with saponification of all fats, neutralization, and then enzymatic determination of glycerol (Triglyceride kit, Sigma) as described previously (Salmon and Flatt, 1985).

10   The assay was carried out for 6-months-old wild-type males (n=4) and *FOXC2* transgenic littermates (n=4) fed ad libitum. The total body lipid content was reduced to 10% of the body weight for transgenic mice compared with the normal 30% of body lipid noted for wild-type mice (Fig. 11).

15

EXAMPLE 7: Lower blood glucose and more efficient glucose elimination in *FOXC2* transgenic mice

Nonfasting blood glucose levels were measured for 10-weeks-old wild-type males and

20   females (n=5+5), and *FOXC2* transgenic littermates (n=5+5) fed ad libitum. The transgenic animals showed a significant 16% reduction in nonfasting blood glucose levels (Fig. 12a).

A glucose tolerance test was performed on 10-weeks-old wild-type males and females

25   (n=5+5) and *FOXC2* transgenic littermates (n=5+5) fed ad libitum. Plasma glucose levels peaked at one minute after intravenous glucose administration, and thereafter plasma glucose levels returned to baseline levels within the 50-minutes study period. Transgenic mice displayed an enhanced glucose elimination, with plasma glucose levels significantly lower than wild-type littermates at 0, 20, and 50 minutes ($P<0.05$;

30     $P<0.001$; $P<0.05$) (Fig. 12b). Data are means of both males and females as no difference between gender was noticed.

39

EXAMPLE 8: Increased insulin sensitivity in *FOXC2* transgenic mice

Non-fasting plasma insulin levels in *FOXC2* transgenic mice were analyzed using the same group of animals as was used for blood glucose measurements. The concentration

5   of plasma insulin in *FOXC2* transgenic mice before the start of the glucose tolerance test were reduced to ~50% of the levels registered for wild-type littermates (Fig. 13a). The rapid intravenous injection of glucose (1 g/kg) raised plasma insulin levels 4-fold in wild-type mice and 10-fold in *FOXC2* transgenic littermates after one minute (Fig. 13c). Thereafter, plasma insulin levels rapidly returned toward baseline values observed

10   before the glucose load, with transgenic mice having significantly lower levels at 20 and 50 minutes ($P < 0.05$; $P < 0.05$) (Fig. 13b). Data are means of both males and females as no difference between gender was noticed.

In mice on a high fat diet, there are significantly lower weight gains in transgenic mice

15   as compared with wt. In females the weight gain is 39% ($p < 0.02$; Fig. 15a) lower as compared with wt females and for males the difference is 21% ($p < 0.03$; Fig. 15b). These findings highlight *FOXC2*, not only as a gene of importance for adipose tissue distribution, morphology and gene expression profile, but also, more importantly, as a major regulator of general lipid and glucose metabolism including protection against

20   diet induced weight gains.

EXAMPLE 9: *FOXC2* regulates body composition, serum lipids and insulin sensitivity

25   Several physiological tests and measurements of critical metabolites with relevance for obesity, insulin resistance and type 2 diabetes were carried out in order to assess the impact of increased *FOXC2* expression on systemic lipid and glucose metabolism. We assayed total body lipid content on whole carcasses using the alcoholic potassium hydroxide digestion method and found a decrease from 30% total lipids in carcasses

30   from wt mice as compared with only 10% in tg mice ($p < 0.0004$; Fig. 16a). There is also a highly significant reduction in serum triglyceride levels by 57% ($p < 0.004$; Fig. 16b), whereas there is no significant change in serum cholesterol levels (Fig. 16c). FFA are lowered from 0.92meq/l to 0,63meq/l in tg animals ($p < 0.02$; Fig. 16d). In *ad libitum* fed

40

animals we note a decrease in plasma glucose levels by 10% ($p < 0.01$; Fig. 16e) and plasma insulin levels are down by 43% ($p < 0.001$; Fig. 16f). In mice on a high fat diet, the weight gain is 28% lower ($p < 0.01$; Fig. 16g) for tg mice as compared with wt litter mates. Although lipid content in tg and wt mice are increased after a high fat diet tg

5   mice still resist lipid accumulation to the same degree as is the case for wt litter mates, a 49% decrease in tg mice as compared with wt ($p < 0.0001$; Fig. 16h). On a control diet (4.8% fat) tg and wt mice display no signs of differences in metabolic efficiency as judged by calculating the ratio between weight gain and weight of food eaten (Fig. 16i). When fed a high fat diet (35.9% fat) tg mice appear less efficient in their metabolism

10   since they need to eat more for each gram of increase in body weight (a reduction by 30% $p < 0.01$; Fig. 16i). These findings are interesting since they suggest that metabolic efficiency, in *FOXC2* tg can be regulated in response to food composition. There are no significant differences in serum glucagon levels, body weight or food consumption (on standard diet) between wt and tg animals (not shown) nor have we observed any

15   significant gender differences.

In an intravenous glucose tolerance test, plasma glucose was assayed at 0, 1, 5, 20, 50 and 75 minutes after glucose administration as depicted in Fig. 17a. Plasma glucose levels for tg mice on control diet are significantly lower at 0, 20 and 75 minutes

20   ($p < 0.05$), and at 50 minutes ($p < 0.02$). The insulin curve for the same experiment exhibit lower insulin levels for tg mice (Fig. 17b) at 0 minutes ($p < 0.02$), at 1, 5 and 20 minutes ($p < 0.05$) and at 50 and 75 minutes ($p < 0.001$). When on a high fat diet the difference after an intravenous glucose tolerance test is much more pronounced (Fig. 17c and d). Glucose levels are lower in tg mice as compared with wt at 0, 5, 20, 50 and 75 minutes

25   ($p < 0.001$; Fig. 17c). Insulin levels are dramatically lowered in tg mice at all time points ($p < 0.001$; Fig. 17d). It is quite extraordinary that tg mice on a high fat diet retain a plasma insulin profile almost identical to that observed when on a standard diet while wt mice display almost a 3-fold increase in insulin plasma levels (Fig. 17 b, d). In spite of this wt mice exhibit a clear increase in glucose levels while tg mice show a much

30   more modest increase in glucose values (Fig. 17 a, b). These findings highlight *FOXC2*, not only as a gene of importance for adipose tissue distribution, morphology and gene expression profile, but also, more importantly, as a major regulator of general lipid and glucose metabolism including protection against diet induced insulin resistance.

41

EXAMPLE 10: Increased sensitivity of the β-adrenergic/cAMP/protein kinase A pathway

5   The induction of the cAMP-regulated *ucp1* in WAT, the hypertrophy of BAT, the decrease of intra-abdominal WAT depots and the up-regulation of mRNAs encoding β-ARs suggest that an increased perturbation and sensitivity in the β-adrenergic/cAMP/protein kinase A (PKA) signaling pathway may contribute to the phenotype of mice over-expressing *FOXC2*. In cotransfection experiments, using 3T3-
10   L1 adipocytes, we show that *FOXC2* increases reporter gene activity of a construct driven by the promoter of the *RIα* gene (encodes the regulatory subunit Iα of PKA; Fig. 18a), whereas no such regulation could be observed for the *RIIβ* promoter (data not shown). Consistent with these findings we also demonstrate increased *RIα* mRNA levels in adipose tissue from tg mice as compared with wt litter mates (Fig. 18a, insert),
15   in a dose dependent manner with regard to transgene expression. This is accompanied by elevated levels of RIα protein and unchanged (WAT) or decreased (BAT) levels of RIIβ protein in tg mice (Fig. 18b). However, basal and total PKA activity is decreased in tg WAT and BAT (Fig. 18c). This may be due to an increased sensitivity to activation by cAMP, dissociation and thereby degradation of PKA holoenzyme. The
20   PKA type I holoenzyme ($RIα_2C_2$) binds cAMP with higher affinity and activates more easily than the PKA type 2 enzyme ($RIIβ_2C_2$), normally expressed in WAT and BAT. Indeed, PKA from WAT of *FOXC2* tg mice with elevated protein levels of RIα (Fig. 18d, insert), activates more easily than PKA from WAT of non-tg litter mates (Fig. 19d, K_{act} of 140 and 250 nM, respectively) although protein levels of RIIβ appear unchanged
25   (Fig. 18d, insert). Thus, adipocytes from *FOXC2* tg mice will have a lower threshold for PKA activation by adrenergic stimuli as compared with wild type litter mates due to a PKA isozyme switch. This is in accordance with what has been reported for mice with targeted disruption of the *RIIβ* gene, where an isozyme-switch from PKA type II ($RIIβ_2C_2$) to PKA type I ($RIα_2C_2$) with a change in K_{act} from 220 nM to 80 nM in WAT
30   leads to a lean mouse with similar phenotype as the *FOXC2* tg mouse. In addition to the increased sensitivity of the PKA-system in *FOXC2* tg adipose tissue, examination of the β-adrenergic sensitivity of WAT-adipocytes from *FOXC2* tg and wt litter mates revealed distinct differences. Whereas stimulation of wt adipocytes with a non-selective

43

β-agonist leads to a 4 to 5-fold increase in cAMP levels at 1 min and a rapid

desensitization of the signal, stimulation of of tg adipocytes leads to a strong (10-fold),

prolonged and sustained increase in cAMP levels which is not desensitized at 10 min

(Fig. 18e). Furthermore, β_3-agonist stimulation of adipocytes prepared from tg WAT

5   displays a distinct increase (4-fold) in cAMP levels with elevated levels over 10 min

(Fig. 19f). In contrast, little or no β_3-agonist response is observed in adipocytes from wt

WAT. These observations are in agreement with the strong up-regulation of β_{1-2}-AR

and the introduction of β_3-AR (which is virtually absent in wt) in tg WAT and indicate

that the sensitivity of β-adrenergic/cAMP/PKA pathway in WAT from *FOXC2* tg mice

10   is increased at several levels.

EXAMPLE 11: Interaction trap / two-hybrid system

15   In order to assay for polypeptides interacting with the FOXC2 polypeptide, the

interaction trap/yeast two-hybrid (Y2H) library screening method is used. This *in vivo*

assay in was first described in Fields & Song (1989) Nature 340, 245-246. A recent

method named Sos recuitment system (SRS) has been described by Aroheim & Karin

(1997) Mol. Cell. Biol. 17, 3094-3102. The SOS system differs from the original yeast

20   2-hybrid for at the interaction between bait and prey takes place in the cytoplasm. A kit

for the SOS-Y2H is available from Stratagene, La Jolla-CA, USA (Cyto TrapTwo-

Hybrid System).

A fusion of an *FOXC2* nucleotide sequence and the human Sos catalytic domain

25   without its C-terminal regulatory domain (Bait) is constructed in an appropriate plasmid

(e.g. pSos) using standard subcloning techniques. Similarly, a myristylation sequence

fusion library (Prey) is constructed in a second plasmid (e.g. pMyr) from cDNA of

potential binding proteins. The Sos fusion construct is verified by sequencing, and

tested for its inability to allow growth of cdc25H yeast strain at the restrictive

30   temperature of 37°C and cell toxicity, both of which would prevent a successful two-

hybrid analysis. The Myr sequence anchors the Myr/library fusion polypeptides to the

plasma membrane and its expression depends on the sugar-containing medium used.

Yeast cells are transformed (ca. 5-10 x 10^6 transformants/mg DNA) with both the Sos

45

and Myr library fusion plasmids according to standard procedures. *In vivo* binding of Sos/(FOXC2) with Myr/library proteins results in a recruitment of the hSos protein to the membrane, thereby activating the Ras-signaling pathway and allowing the cdc25H yeast cells to grow at 37°C. Yeast cells are first plated on a glucose media lacking the amino acids Leu and Ura to select both plasmids. After 4-5 days at 25°C, plates are used for replica plating onto galactose (-Leu, -Ura) plates incubated at 37°C for 3-10 days . Colonies that are growing are first tested for their ability to grow at 37°C depending of the sugar present in the medium. Those clones that show growth in galactose compared to glucose are considered candidates. To test the specificity of the of the library plasmids, plasmid DNA is extracted from candidate clones and used to cotransform cdc25H cells with either the specific bait or a non-relevant bait. Insert DNA is sequenced to verify the presence of an open reading frame fused to the Myr sequence and to determine the identity of the interacting protein.

EXAMPLE 12: Mobility shift DNA-binding assay

A gel electrophoresis mobility shift assay, according to standard procedures, can rapidly detect specific protein-DNA interactions. Probe DNA (<300 bp) is obtained from synthetic oligonucleotides, restriction endonuclease fragments, or PCR fragments and end-labeled with ^{32}P. An aliquot of purified FOXC2 (ca. 15 µg) or crude FOXC2 extract (ca. 15 ng) is incubated at constant temperature (in the range 22–37°C) for at least 30 minutes in 10-15 µl of buffer (i.e. TAE or TBE, pH 8.0-8.5) containing radiolabeled probe DNA, nonspecific carrier DNA (ca. 1 µg), BSA (300 µg/ml), and 10% (v/v) glycerol. The reaction mixture is then loaded onto a polyacrylamide gel and run at 30-35 mA until good separation of free probe DNA from protein-DNA complexes occurs. The gel is then dried and bands corresponding to free DNA and protein-DNA complexes are detected by autoradiography.

44

EXAMPLE 13: Reporter gene assay to identify modulating compounds

Reporter gene assays are well known as tools to signal transcriptional activity in cells.
(For a review of chemiluminescent and bioluminescent reporter gene assays, see

5   Bronstein et al. (1994) Analytical Biochemistry 219, 169-181.) For instance, the
photoprotein luciferase provides a useful tool for assaying for modulators of FOXC2
activity. Cells (e.g., CHO cells or COS 7 cells) are transiently co-transfected with both a
FOXC2 expression construct and a reporter construct which includes a gene for the
luciferase protein downstream from a transcription factor binding site. Agonist binding

10   to FOXC2 results in expression of the luciferase gene. Luciferase activity may be
quantitatively measured using e.g. luciferase assay reagents that are commercially
available from Promega (Madison, WI). Differences in luminescence in the presence
versus the absence of a candidate modulator compound are indicative of modulatory
activity.

15

EXAMPLE 14: Expressing profiling and target identification using microarrays

To better understand the role of FOXC2 and thus identify new drug targets, the

20   expression patterns of a large number of genes and ESTs can be monitored using
GeneChip® expression microarrays (http://www.affymetrix.com/products/
app_exp.html). Microarrays consist of a highly ordered matrix of thousands of different
sequences that can be used to measure DNA and RNA variation in applications that
include gene expression profiling, comparative genomics and genotyping (for recent

25   reviews, see e.g.: Harrington et al. (2000) Monitoring gene expression using DNA
microarrays. Curr. Opin. Microbiol. 3(3): 285-291; or Dugan et al. (1999) Expression
profiling using cDNA microarrays. Nature Genetics supplement 21:10-14).

Briefly, RNAs are extracted from any tissues or cultured cells relevant for the study of

30   FOXC2 and reverse transcribed using a T7-tagged oligo-dT primer to generate double-
stranded cDNAs. These cDNAs are then amplified and labeled using In Vitro
Transcription (IVT) with T7 RNA polymerase and biotinylated nucleotides. The
populations of cRNAs obtained after IVT are purified and fragmented by heat to

45

produce a distribution of RNA fragment sizes from approximately 35 to 200 bases. GeneChip® expression arrays are hybridized with the samples. The arrays are washed and stained. The cartridges are scanned using a confocal scanner and the images are analyzed with the Microarray Suite 4.0 software (Affymetrix).

5

Using this analysis the role of FOXC2 in specific signaling pathways can be elucidated, and new signaling pathways can be identified. Genes directly or indirectly regulated by FOXC2 can be identified. The analysis will identify genes with previously known function but also novel genes. Gene products functioning in the same pathways as

10　FOXC2 can be considered putative target proteins. Targeting of such proteins would be expected to yield some or all of the desired effects expected from targeting FOXC2 directly.

15　**REFERENCES**

Ailhaud, G., Grimaldi, P., and Negrel, R. (1992). Cellular and molecular aspects of adipose tissue development. *Annu Rev Nutr* **12**, 207-33.

20　Arch, J. R., and Wilson, S. (1996). Prospects for beta 3-adrenoceptor agonists in the treatment of obesity and diabetes. *Int J Obes Relat Metab Disord* **20**, 191-9.

Barak, Y., Nelson, M. C., Ong, E. S., Jones, Y. Z., Ruiz-Lozano, P., Chien, K. R., Koder, A., and Evans, R. M. (1999). PPAR gamma is required for placental, cardiac,

25　and adipose tissue development. *Mol Cell* **4**, 585-95.

Bloom, J. D., Dutia, M. D., Johnson, B. D., Wissner, A., Burns, M. G., Largis, E. E., Dolan, J. A., and Claus, T. H. (1992). Disodium (R,R)-5-[2-[[2-(3-chlorophenyl)-2-hydroxyethyl]-amino] propyl]- 1,3-benzodioxole-2,2-dicarboxylate (CL 316,243). A

30　potent beta- adrenergic agonist virtually specific for beta 3 receptors. A promising antidiabetic and antiobesity agent. *J Med Chem* **35**, 3081-4.

Boss, O., Samec, S., Paoloni-Giacobino, A., Rossier, C., Dulloo, A., Seydoux, J., Muzzin, P., and Giacobino, J. P (1997). Uncoupling protein-3: a new member of the mitochondrial carrier family with tissue-specific expression. *FEBS Lett* **408**, 39-42.

5   Brewer, C. B. (1994). Cytomegalovirus plasmid vectors for permanent lines of polarized epithelial cells. *Methods Cell Biol* **43**, 233-45.

Brown, M. S., and Goldstein, J. L. (1997). The SREBP pathway: regulation of cholesterol metabolism by proteolysis of a membrane-bound transcription factor. *Cell*
10 **89**, 331-40.

Cao, Z., Umek, R. M., and McKnight, S. L. (1991). Regulated expression of three C/EBP isoforms during adipose conversion of 3T3-L1 cells. *Genes Dev* **5**, 1538-52.

15 Cawthorne, M. A., Sennitt, M. V., Arch, J. R., and Smith, S. A. (1992). BRL 35135, a potent and selective atypical beta-adrenoceptor agonist. *Am J Clin Nutr* **55**, 252S-257S.

Cederberg, A., Betz, R., Lagercrantz, S., Larsson, C., Hulander, M., Carlsson, P., and Enerback, S. (1997). Chromosome localization, sequence analysis, and expression
20 pattern identify FKHL 18 as a novel human forkhead gene. *Genomics* **44**, 344-6.

Champigny, O., and Ricquier, D. (1996). Evidence from *in vitro* differentiating cells that adrenoceptor agonists can increase uncoupling protein mRNA level in adipocytes of adult humans: an RT-PCR study. *J Lipid Res* **37**, 1907-14.

25
Choy, L. N., and Spiegelman, B. M. (1996). Regulation of alternative pathway activation and C3a production by adipose cells. *Obes Res* **4**, 521-32.

Christy, R. J., Yang, V. W., Ntambi, J. M., Geiman, D. E., Landschulz, W. H.,
30 Friedman, A. D., Nakabeppu, Y., Kelly, T. J., and Lane, M. D. (1989). Differentiation-induced gene expression in 3T3-L1 preadipocytes: CCAAT/enhancer binding protein interacts with and activates the promoters of two adipocyte-specific genes. *Genes Dev* **3**, 1323-35.

47

Chua, S. C., Jr., Chung, W. K., Wu-Peng, X. S., Zhang, Y., Liu, S. M., Tartaglia, L., and Leibel, R. L. (1996). Phenotypes of mouse diabetes and rat fatty due to mutations in the OB (leptin) receptor. *Science* **271**, 994-6.

Cianflone, K., Maslowska, M., and Sniderman, A. (1995). The acylation stimulating protein-adipsin system. *Int J Obes Relat Metab Disord* **19 Suppl 1**, S34-8.

Coleman, D. L. (1973). Effects of parabiosis of obese with diabetes and normal mice. *Diabetologia* **9**, 294-8.

Cornelius, P., MacDougald, O. A., and Lane, M. D. (1994). Regulation of adipocyte development. *Annu Rev Nutr* **14**, 99-129.

Cousin, B., Cinti, S., Morroni, M., Raimbault, S., Ricquier, D., Penicaud, L., and Casteilla, L. (1992). Occurrence of brown adipocytes in rat white adipose tissue: molecular and morphological characterization. *J Cell Sci* **103**, 931-42.

Cummings, D. E., Brandon, E. P., Planas, J. V., Motamed, K., Idzerda, R. L., and McKnight, G. S. (1996). Genetically lean mice result from targeted disruption of the RII beta subunit of protein kinase A. *Nature* **382**, 622-6.

De Vos, P., Lefebvre, A. M., Miller, S. G., Guerre-Millo, M., Wong, K., Saladin, R., Hamann, L. G., Staels, B., Briggs, M. R., and Auwerx, J. (1996). Thiazolidinediones repress ob gene expression in rodents via activation of peroxisome proliferator-activated receptor gamma. *J Clin Invest* **98**, 1004-9.

Digby, J. E., Montague, C. T., Sewter, C. P., Sanders, L., Wilkison, W. O., O'Rahilly, S., and Prins, J. B. (1998). Thiazolidinedione exposure increases the expression of uncoupling protein 1 in cultured human preadipocytes. *Diabetes* **47**, 138-41.

Elchebly, M., Payette, P., Michaliszyn, E., Cromlish, W., Collins, S., Loy, A. L., Normandin, D., Cheng, A., Himms-Hagen, J., Chan, C. C., Ramachandran, C., Gresser, M. J., Tremblay, M. L., and Kennedy, B. P. (1999). Increased insulin sensitivity and

4*f*

obesity resistance in mice lacking the protein tyrosine phosphatase-1B gene. *Science*
283, 1544-8.

Enerback, S., Jacobsson, A., Simpson, E. M., Guerra, C., Yamashita, H., Harper, M. E.,
and Kozak, L. P. (1997). Mice lacking mitochondrial uncoupling protein are cold-
sensitive but not obese. *Nature* **387**, 90-4.

Enerback, S., Ohlsson, B. G., Samuelsson, L., and Bjursell, G. (1992). Characterization
of the human lipoprotein lipase (LPL) promoter: evidence of two cis-regulatory regions,
LP-alpha and LP-beta, of importance for the differentiation-linked induction of the LPL
gene during adipogenesis. *Mol Cell Biol* **12**, 4622-33.

Fajas, L., Auboeuf, D., Raspe, E., Schoonjans, K., Lefebvre, A. M., Saladin, R., Najib,
J., Laville, M., Fruchart, J. C., Deeb, S., Vidal-Puig, A., Flier, J., Briggs, M. R., Staels,
B., Vidal, H., and Auwerx, J. (1997). The organization, promoter analysis, and
expression of the human PPARgamma gene. *J Biol Chem* **272**, 18779-89.

Fajas, L., Schoonjans, K., Gelman, L., Kim, J. B., Najib, J., Martin, G., Fruchart, J. C.,
Briggs, M., Spiegelman, B. M., and Auwerx, J. (1999). Regulation of peroxisome
proliferator-activated receptor gamma expression by adipocyte differentiation and
determination factor 1/sterol regulatory element binding protein 1: implications for
adipocyte differentiation and metabolism. *Mol Cell Biol* **19**, 5495-503.

Fleury, C., Neverova, M., Collins, S., Raimbault, S., Champigny, O., Levi-Meyrueis,
C., Bouillaud, F., Seldin, M. F., Surwit, R. S., Ricquier, D., and Warden, C. H. (1997).
Uncoupling protein-2: a novel gene linked to obesity and hyperinsulinemia [see
comments]. *Nat Genet* **15**, 269-72.

Forman, B. M., Tontonoz, P., Chen, J., Brun, R. P., Spiegelman, B. M., and Evans, R.
M. (1995). 15-Deoxy-delta 12, 14-prostaglandin J2 is a ligand for the adipocyte
determination factor PPAR gamma. *Cell* **83**, 803-12.

Furumoto, T. A., Miura, N., Akasaka, T., Mizutani-Koseki, Y., Sudo, H., Fukuda, K.,
Maekawa, M., Yuasa, S., Fu, Y., Moriya, H., Taniguchi, M., Imai, K., Dahl, E., Balling,

49

R., Pavlova, M., Gossler, A., and Koseki, H. (1999). Notochord-dependent expression of MFH1 and PAX1 cooperates to maintain the proliferation of sclerotome cells during the vertebral column development. *Dev Biol* **210**, 15-29.

Ghorbani, M., Claus, T. H., and Himms-Hagen, J. (1997). Hypertrophy of brown adipocytes in brown and white adipose tissues and reversal of diet-induced obesity in rats treated with a beta3- adrenoceptor agonist. *Biochem Pharmacol* **54**, 121-31.

Giacobino, J. P. (1995). Beta 3-adrenoceptor: an update. *Eur J Endocrinol* **132**, 377-85.

Granneman, J. G., and Lahners, K. N. (1994). Analysis of human and rodent beta 3-adrenergic receptor messenger ribonucleic acids. *Endocrinology* **135**, 1025-31.

Granneman, J. G., Lahners, K. N., and Chaudhry, A. (1991). Molecular cloning and expression of the rat beta 3-adrenergic receptor. *Mol Pharmacol* **40**, 895-9.

Himms-Hagen, J. (1989). Brown adipose tissue thermogenesis and obesity. *Prog Lipid Res* **28**, 67-115.

Himms-Hagen, J., Cui, J., Danforth, E., Jr., Taatjes, D. J., Lang, S. S., Waters, B. L., and Claus, T. H. (1994). Effect of CL-316,243, a thermogenic beta 3-agonist, on energy balance and brown and white adipose tissues in rats. *Am J Physiol* **266**, R1371-82.

Hofmann, C., Lorenz, K., Braithwaite, S. S., Colca, J. R., Palazuk, B. J., Hotamisligil, G. S., and Spiegelman, B. M. (1994). Altered gene expression for tumor necrosis factor-alpha and its receptors during drug and dietary modulation of insulin resistance. *Endocrinology* **134**, 264-70.

Holloway, B. R., Howe, R., Rao, B. S., and Stribling, D. (1992). ICI D7114: a novel selective adrenoceptor agonist of brown fat and thermogenesis. *Am J Clin Nutr* **55**, 262S-264S.

50

Hotamisligil, G. S., Arner, P., Caro, J. F., Atkinson, R. L., and Spiegelman, B. M. (1995). Increased adipose tissue expression of tumor necrosis factor-alpha in human obesity and insulin resistance. *J Clin Invest* **95**, 2409-15.

5   Hotamisligil, G. S., Shargill, N. S., and Spiegelman, B. M. (1993). Adipose expression of tumor necrosis factor-alpha: direct role in obesity-linked insulin resistance. *Science* **259**, 87-91.

Iida, K., Koseki, H., Kakinuma, H., Kato, N., Mizutani-Koseki, Y., Ohuchi, H.,
10 Yoshioka, H., Noji, S., Kawamura, K., Kataoka, Y., Ueno, F., Taniguchi, M., Yoshida, N., Sugiyama, T., and Miura, N. (1997). Essential roles of the winged helix transcription factor MFH-1 in aortic arch patterning and skeletogenesis. *Development* **124**, 4627-38.

15 Ito, M., Grujic, D., Abel, E. D., Vidal-Puig, A., Susulic, V. S., Lawitts, J., Harper, M. E., Himms-Hagen, J., Strosberg, A. D., and Lowell, B. B. (1998). Mice expressing human but not murine beta3-adrenergic receptors under the control of human gene regulatory elements. *Diabetes* **47**, 1464-71.

20 Jezek, P., Orosz, D. E., Modriansky, M., and Garlid, K. D. (1994). Transport of anions and protons by the mitochondrial uncoupling protein and its regulation by nucleotides and fatty acids. A new look at old hypotheses. *J Biol Chem* **269**, 26184-90.

Kaestner, K. H., Bleckmann, S. C., Monaghan, A. P., Schlondorff, J., Mincheva, A.,
25 Lichter, P., and Schutz, G. (1996). Clustered arrangement of winged helix genes fkh-6 and MFH-1: possible implications for mesoderm development. *Development* **122**, 1751-8.

Kaestner, K. H., Christy, R. J., and Lane, M. D. (1990). Mouse insulin-responsive
30 glucose transporter gene: characterization of the gene and trans-activation by the CCAAT/enhancer binding protein. *Proc Natl Acad Sci U S A* **87**, 251-5.

5 1

Kaestner, K. H., Lee, K. H., Schlondorff, J., Hiemisch, H., Monaghan, A. P., and Schutz, G. (1993). Six members of the mouse forkhead gene family are developmentally regulated. *Proc Natl Acad Sci U S A* **90**, 7628-31.

5 Kim, J. B., and Spiegelman, B. M. (1996). ADD1/SREBP1 promotes adipocyte differentiation and gene expression linked to fatty acid metabolism. *Genes Dev* **10**, 1096-107.

Kim, J. B., Wright, H. M., Wright, M., and Spiegelman, B. M. (1998). ADD1/SREBP1
10 activates PPARgamma through the production of endogenous ligand. *Proc Natl Acad Sci U S A* **95**, 4333-7.

Klaus, S. (1997). Functional differentiation of white and brown adipocytes. *Bioessays* **19**, 215-23.

15

Kopecky, J., Clarke, G., Enerback, S., Spiegelman, B., and Kozak, L. P. (1995). Expression of the mitochondrial uncoupling protein gene from the aP2 gene promoter prevents genetic obesity. *J Clin Invest* **96**, 2914-23.

20 Kozak, L. P., Kozak, U. C., and Clarke, G. T. (1991). Abnormal brown and white fat development in transgenic mice overexpressing glycerol 3-phosphate dehydrogenase. *Genes Dev* **5**, 2256-64.

Krief, S., Lonnqvist, F., Raimbault, S., Baude, B., Van Spronsen, A., Arner, P.,
25 Strosberg, A. D., Ricquier, D., and Emorine, L. J. (1993). Tissue distribution of beta 3-adrenergic receptor mRNA in man. *J Clin Invest* **91**, 344-9.

Lafontan, M., and Berlan, M. (1993). Fat cell adrenergic receptors and the control of white and brown fat cell function. *J Lipid Res* **34**, 1057-91.

30

Lean, M. E., James, W. P., Jennings, G., and Trayhurn, P. (1986). Brown adipose tissue uncoupling protein content in human infants, children and adults. *Clin Sci* **71**, 291-7.

Lehmann, J. M., Moore, L. B., Smith-Oliver, T. A., Wilkison, W. O., Willson, T. M., and Kliewer, S. A. (1995). An antidiabetic thiazolidinedione is a high affinity ligand for peroxisome proliferator-activated receptor gamma (PPAR gamma). *J Biol Chem* **270**, 12953-6.

Lin, F. T., and Lane, M. D. (1992). Antisense CCAAT/enhancer-binding protein RNA suppresses coordinate gene expression and triglyceride accumulation during differentiation of 3T3- L1 preadipocytes. *Genes Dev* **6**, 533-44.

Lin, F. T., and Lane, M. D. (1994). CCAAT/enhancer binding protein alpha is sufficient to initiate the 3T3- L1 adipocyte differentiation program. *Proc Natl Acad Sci U S A* **91**, 8757-61.

Loncar, D. (1991). Convertible adipose tissue in mice. *Cell Tissue Res* **266**, 149-61.

Lopez, J. M., Bennett, M. K., Sanchez, H. B., Rosenfeld, J. M., and Osborne, T. E. (1996). Sterol regulation of acetyl coenzyme A carboxylase: a mechanism for coordinate control of cellular lipid. *Proc Natl Acad Sci U S A* **93**, 1049-53.

Lowell, B. B., V, S. S., Hamann, A., Lawitts, J. A., Himms-Hagen, J., Boyer, B. B., Kozak, L. P., and Flier, J. S. (1993). Development of obesity in transgenic mice after genetic ablation of brown adipose tissue. *Nature* **366**, 740-2.

MacDougald, O. A., Cornelius, P., Lin, F. T., Chen, S. S., and Lane, M. D. (1994). Glucocorticoids reciprocally regulate expression of the CCAAT/enhancer- binding protein alpha and delta genes in 3T3-L1 adipocytes and white adipose tissue. *J Biol Chem* **269**, 19041-7.

Mantzoros, C. S., Qu, D., Frederich, R. C., Susulic, V. S., Lowell, B. B., Maratos-Flier, E., and Flier, J. S. (1996). Activation of beta(3) adrenergic receptors suppresses leptin expression and mediates a leptin-independent inhibition of food intake in mice. *Diabetes* **45**, 909-14.

5 3

Martin, G., Schoonjans, K., Lefebvre, A. M., Staels, B., and Auwerx, J. (1997). Coordinate regulation of the expression of the fatty acid transport protein and acyl-CoA synthetase genes by PPARalpha and PPARgamma activators. *J Biol Chem* **272**, 28210-7.

Maslowska, M., Sniderman, A. D., Germinario, R., and Cianflone, K. (1997). ASP stimulates glucose transport in cultured human adipocytes. *Int J Obes Relat Metab Disord* **21**, 261-6.

Miller, C. W., and Ntambi, J. M. (1996). Peroxisome proliferators induce mouse liver stearoyl-CoA desaturase 1 gene expression. *Proc Natl Acad Sci U S A* **93**, 9443-8.

Miura, N., Wanaka, A., Tohyama, M., and Tanaka, K. (1993). MFH-1, a new member of the fork head domain family, is expressed in developing mesenchyme. *FEBS Lett* **326**, 171-6.§

Muzzin, P., Revelli, J. P., Kuhne, F., Gocayne, J. D., McCombie, W. R., Venter, J. C., Giacobino, J. P., and Fraser, C. M. (1991). An adipose tissue-specific beta-adrenergic receptor. Molecular cloning and down-regulation in obesity. *J Biol Chem* **266**, 24053-8.

Nahmias, C., Blin, N., Elalouf, J. M., Mattei, M. G., Strosberg, A. D., and Emorine, L. J. (1991). Molecular characterization of the mouse beta 3-adrenergic receptor: relationship with the atypical receptor of adipocytes. *Embo J* **10**, 3721-7.

Nicholls, D. G., and Locke, R. M. (1984). Thermogenic mechanisms in brown fat. *Physiol Rev* **64**, 1-64.

Park, E. A., Roesler, W. J., Liu, J., Klemm, D. J., Gurney, A. L., Thatcher, J. D., Shuman, J., Friedman, A., and Hanson, R. W. (1990). The role of the CCAAT/enhancer-binding protein in the transcriptional regulation of the gene for phosphoenolpyruvate carboxykinase (GTP). *Mol Cell Biol* **10**, 6264-72.

Peraldi, P., Xu, M., and Spiegelman, B. M. (1997). Thiazolidinediones block tumor necrosis factor-alpha-induced inhibition of insulin signaling. *J Clin Invest* **100**, 1863-9.

5 4

Petruschke, T., and Hauner, H. (1993). Tumor necrosis factor-alpha prevents the differentiation of human adipocyte precursor cells and causes delipidation of newly developed fat cells. *J Clin Endocrinol Metab* **76**, 742-7.

Pierrou, S., Hellqvist, M., Samuelsson, L., Enerback, S., and Carlsson, P. (1994). Cloning and characterization of seven human forkhead proteins: binding site specificity and DNA bending. *Embo J* **13**, 5002-12.

Puigserver, P., Wu, Z., Park, C. W., Graves, R., Wright, M., and Spiegelman, B. M. (1998). A cold-inducible coactivator of nuclear receptors linked to adaptive thermogenesis. *Cell* **92**, 829-39.

Rehnmark, S., Nechad, M., Herron, D., Cannon, B., and Nedergaard, J. (1990). Alpha- and beta-adrenergic induction of the expression of the uncoupling protein thermogenin in brown adipocytes differentiated in culture. *J Biol Chem* **265**, 16464-71.

Ricquier, D., Bouillaud, F., Toumelin, P., Mory, G., Bazin, R., Arch, J., and Penicaud, L. (1986). Expression of uncoupling protein mRNA in thermogenic or weakly thermogenic brown adipose tissue. Evidence for a rapid beta- adrenoreceptor-mediated and transcriptionally regulated step during activation of thermogenesis. *J Biol Chem* **261**, 13905-10.

Ross, S. R., Graves, R. A., Greenstein, A., Platt, K. A., Shyu, H. L., Mellovitz, B., and Spiegelman, B. M. (1990). A fat-specific enhancer is the primary determinant of gene expression for adipocyte P2 *in vivo*. *Proc Natl Acad Sci U S A* **87**, 9590-4.

Salmon, D. M., and Flatt, J. P. (1985). Effect of dietary fat content on the incidence of obesity among ad libitum fed mice. *Int J Obes* **9**, 443-9.

Samuelsson, L., Stromberg, K., Vikman, K., Bjursell, G., and Enerback, S. (1991). The CCAAT/enhancer binding protein and its role in adipocyte differentiation: evidence for direct involvement in terminal adipocyte development. *EMBO J* **10**, 3787-93.

55

Schoonjans, K., Peinado-Onsurbe, J., Lefebvre, A. M., Heyman, R. A., Briggs, M., Deeb, S., Staels, B., and Auwerx, J. (1996). PPARalpha and PPARgamma activators direct a distinct tissue-specific transcriptional response via a PPRE in the lipoprotein lipase gene. *EMBO J* **15**, 5336-48.

5

Sethi, J. K., and Hotamisligil, G. S. (1999). The role of TNF alpha in adipocyte metabolism. *Semin Cell Dev Biol* **10**, 19-29.

Shimomura, I., Hammer, R. E., Richardson, J. A., Ikemoto, S., Bashmakov, Y.,

10   Goldstein, J. L., and Brown, M. S. (1998). Insulin resistance and diabetes mellitus in transgenic mice expressing nuclear SREBP-1c in adipose tissue: model for congenital generalized lipodystrophy. *Genes Dev* **12**, 3182-94.

Soloveva, V., Graves, R. A., Rasenick, M. M., Spiegelman, B. M., and Ross, S. R.

15   (1997). Transgenic mice overexpressing the beta 1-adrenergic receptor in adipose tissue are resistant to obesity. *Mol Endocrinol* **11**, 27-38.

Susulic, V. S., Frederich, R. C., Lawitts, J., Tozzo, E., Kahn, B. B., Harper, M. E., Himms-Hagen, J., Flier, J. S., and Lowell, B. B. (1995). Targeted disruption of the beta

20   3-adrenergic receptor gene. *J Biol Chem* **270**, 29483-92.

Tanaka, T., Yoshida, N., Kishimoto, T., and Akira, S. (1997). Defective adipocyte differentiation in mice lacking the C/EBPbeta and/or C/EBPdelta gene. *EMBO J* **16**, 7432-43.

25

Tontonoz, P., Hu, E., Devine, J., Beale, E. G., and Spiegelman, B. M. (1995). PPAR gamma 2 regulates adipose expression of the phosphoenolpyruvate carboxykinase gene. *Mol Cell Biol* **15**, 351-7.

30   Tontonoz, P., Hu, E., Graves, R. A., Budavari, A. I., and Spiegelman, B. M. (1994a). mPPAR gamma 2: tissue-specific regulator of an adipocyte enhancer. *Genes Dev* **8**, 1224-34.

56

Tontonoz, P., Hu, E., and Spiegelman, B. M. (1994b). Stimulation of adipogenesis in fibroblasts by PPAR gamma 2, a lipid- activated transcription factor [published erratum appears in Cell 1995 Mar 24;80(6):following 957]. *Cell* **79**, 1147-56.

5   Walsh, M. J., Sniderman, A. D., Cianflone, K., Vu, H., Rodriguez, M. A., and Forse, R. A. (1989). The effect of ASP on the adipocyte of the morbidly obese. *J Surg Res* **46**, 470-3.

Wang, N. D., Finegold, M. J., Bradley, A., Ou, C. N., Abdelsayed, S. V., Wilde, M. D.,

10   Taylor, L. R., Wilson, D. R., and Darlington, G. J. (1995). Impaired energy homeostasis in C/EBP alpha knockout mice. *Science* **269**, 1108-12.

Werman, A., Hollenberg, A., Solanes, G., Bjorbaek, C., Vidal-Puig, A. J., and Flier, J. S. (1997). Ligand-independent activation domain in the N terminus of peroxisome

15   proliferator-activated receptor gamma (PPARgamma). Differential activity of PPARgamma1 and -2 isoforms and influence of insulin. *J Biol Chem* **272**, 20230-5.

Wickelgren, I. (1998). Obesity: how big a problem? [news]. *Science* **280**, 1364-7.

20   Winnier, G. E., Hargett, L., and Hogan, B. L. (1997). The winged helix transcription factor MFH1 is required for proliferation and patterning of paraxial mesoderm in the mouse embryo. *Genes Dev* **11**, 926-40.

Wu, Z., Bucher, N. L., and Farmer, S. R. (1996). Induction of peroxisome proliferator-

25   activated receptor gamma during the conversion of 3T3 fibroblasts into adipocytes is mediated by C/EBPbeta, C/EBPdelta, and glucocorticoids. *Mol Cell Biol* **16**, 4128-36.

Wu, Z., Puigserver, P., Andersson, U., Zhang, C., Adelmant, G., Mootha, V., Troy, A., Cinti, S., Lowell, B., Scarpulla, R. C., and Spiegelman, B. M. (1999a). Mechanisms

30   controlling mitochondrial biogenesis and respiration through the thermogenic coactivator PGC-1. *Cell* **98**, 115-24.

Wu, Z., Rosen, E. D., Brun, R., Hauser, S., Adelmant, G., Troy, A. E., McKeon, C., Darlington, G. J., and Spiegelman, B. M. (1999b). Cross-regulation of C/EBP alpha and

57

PPAR gamma controls the transcriptional pathway of adipogenesis and insulin sensitivity. *Mol Cell* **3**, 151-8.

Wu, Z., Xie, Y., Bucher, N. L., and Farmer, S. R. (1995). Conditional ectopic
5 expression of C/EBP beta in NIH-3T3 cells induces PPAR gamma and stimulates adipogenesis. *Genes Dev* **9**, 2350-63.

Yeh, W. C., Cao, Z., Classon, M., and McKnight, S. L. (1995). Cascade regulation of terminal adipocyte differentiation by three members of the C/EBP family of leucine
10 zipper proteins. *Genes Dev* **9**, 168-81.

Yubero, P., Manchado, C., Cassard-Doulcier, A. M., Mampel, T., Vinas, O., Iglesias, R., Giralt, M., and Villarroya, F. (1994). CCAAT/enhancer binding proteins alpha and beta are transcriptional activators of the brown fat uncoupling protein gene promoter.
15 *Biochem Biophys Res Commun* **198**, 653-9.

Zhang, Y., Proenca, R., Maffei, M., Barone, M., Leopold, L., and Friedman, J. M. (1994). Positional cloning of the mouse obese gene and its human homologue [published erratum appears in Nature 1995 Mar 30;374(6521):479]. *Nature* **372**, 425-
20 32.

5𝑑

CLAIMS

1.   A construct comprising a human *FOXC2* nucleotide sequence operably linked to an element selected from the group consisting of promoters, response elements, enhancer elements and mixtures thereof.

2.   The construct according to claim 1 wherein the said element is an adipose-specific promoter.

3.   The construct according to claim 2 wherein the said adipose-specific promoter is the promoter of the murine gene encoding adipocyte P2.

4.   The construct according to any one of claims 1 to 3 wherein the said human *FOXC2* nucleotide sequence is selected from:
      (a) the nucleotide sequence shown as SEQ ID NO: 1;
      (b) nucleotide sequences capable of hybridizing, under stringent hybridization conditions, to a nucleotide sequence complementary to the polypeptide coding region of a nucleotide sequence as defined in (a) and which codes for a biologically active FOXC2 polypeptide, or a functionally equivalent modified form thereof;
      (c) nucleic acid sequence which are degenerate as a result of the genetic code to a nucleotide sequence as defined in (a) or (b) and which codes for a biologically active FOXC2 polypeptide, or a functionally equivalent modified form thereof.

5.   A transgenic non-human mammalian animal whose genome comprises a gene construct according to any one of claims 1 to 4, said animal being capable of expressing the human *FOXC2* gene in its adipose tissue.

6.   A transgenic non-human mammalian animal according to claim 5 which is a mouse.

7.   A transgenic mouse according to claim 6 which is derived from a genetically obese mouse.

59

8.   An isolated cell line derived from the transgenic non-human mammalian animal according to any one of claims 5 to 7.

5   9.   A method for producing a transgenic non-human mammalian animal overexpressing the human *FOXC2* gene, said method comprising chromosomally incorporating a gene construct according to any one of claims 1 to 4 into the genome of said non-human mammalian animal.

10   10.   A method for studying the biological activity of a polypeptide encoded by the human *FOXC2* gene, said method comprising the steps
(i) producing a transgenic non-human mammalian animal according to the method of claim 9; and
(ii) comparing the phenotype of the said transgenic non-human mammalian
15   animal with a wild-type animal of the same species.

11.   A method for identifying a compound useful for the treatment of a medical condition related to obesity, said method comprising the steps
(i) contacting a test compound with the human *FOXC2* gene; and
20   (ii) determining whether said test compound activates the expression of the human *FOXC2* gene, such activation being indicative for a compound useful for the treatment of a medical condition related to obesity.

12.   A method of screening for a compound useful for the treatment of a medical
25   condition related to obesity, said method comprising exposing a non-human mammalian animal to a test compound, and determining the activity of the human *FOXC2* gene in said non-human mammalian animal, wherein an increase in said gene activity as compared to an untreated non-human mammalian animal being indicative of a compound useful for the treatment of a medical condition related to
30   obesity.

13.   The method according to claim 12 wherein the said non-human mammalian animal is a mouse.

14. The method according to claim 13 wherein the said mouse is a genetically obese mouse.

15. The method according to any one of claims 12 to 14, wherein the activity of said human *FOXC2* gene in said non-human mammalian animal, is compared to the activity of the said human *FOXC2* gene in a transgenic non-human mammalian animal expressing the said human *FOXC2* gene.

16. A method for identifying a compound useful for the treatment of a medical condition related to obesity, said method comprising the steps
    (i) contacting a test compound with a polypeptide encoded by the human *FOXC2* gene; and
    (ii) determining whether said test compound stimulates the biological activities of the said polypeptide, such stimulation being indicative for a compound useful for the treatment of a medical condition related to obesity.

17. Use of a compound identified by the method according to any one of claims 11 to 16 in the manufacture of a medicament for the treatment of a medical condition related to obesity.

18. A method for the treatment of a medical condition related to obesity, comprising administering to a subject an effective amount of a compound identified by the method according to any one of claims 11 to 16.

19. The method or use according to any one of claims 11 to 18, wherein the said medical condition related to obesity is selected from obesity, NIDDM, hypertension and hyperlipidemia.

20. A method for identifying a compound useful for the treatment of a medical condition related to malnutrition, said method comprising the steps
    (i) contacting a test compound with the human *FOXC2* gene; and
    (ii) determining whether said test compound decreases expression of the *FOXC2* gene, such decrease being indicative for a compound useful for the treatment of a medical condition related to malnutrition.

61

21. A method of screening for a compound useful for the treatment of a medical condition related to malnutrition, said method comprising exposing a non-human mammalian animal to a test compound, and determining the activity of said human *FOXC2* gene in said non-human mammalian animal, wherein a decrease in said gene activity as compared to an untreated non-human mammalian animal being indicative of a compound useful for the treatment of a medical condition related to malnutrition.

22. The method according to claim 21 wherein the said non-human mammalian animal is a mouse.

23. The method according to claim 22 wherein the said mouse is a genetically obese mouse.

24. The method according to any one of claims 21 to 23, wherein the activity of said human *FOXC2* gene in said non-human mammalian animal, is compared to the activity of the said human *FOXC2* gene in a transgenic non-human mammalian animal expressing the said human *FOXC2* gene.

25. A method for identifying a compound useful for the treatment of a medical condition related to malnutrition, said method comprising the steps
(i) contacting a test compound with a polypeptide encoded by the human *FOXC2* gene; and
(ii) determining whether said test compound decreases the biological activities of the said polypeptide, such decrease being indicative for a compound useful for the treatment of a medical condition related to malnutrition.

26. Use of a compound identified by the method according to any one of claims 20 to 25 in the manufacture of a medicament for the treatment of a medical condition related to malnutrition.

62

27. A method for the treatment of a medical condition related to malnutrition, comprising administering to a subject an effective amount of a compound identified by the method according to any one of claims 20 to 25.

28. The method or use according to any one of claims 20 to 27, wherein the said medical condition related to malnutrition is anorexia.

29. Use of a polypeptide encoded by the human *FOXC2* gene in the manufacture of a medicament for the treatment of a medical condition related to obesity.

30. A method for the treatment of a medical condition related to obesity, comprising administering to a subject an effective amount of a polypeptide encoded by the human *FOXC2* gene.

31. A vector comprising a construct according to any one of claims 1 to 4.

32. A method of treating obesity in a human, comprising
(i) administering to the said human a vector according to claim 31; and
(ii) allowing the said human to express a therapeutically effective amount of a polypeptide encoded by the human *FOXC2* gene.

33. The method according to claim 32 wherein the said vector is derived from an adenoviral vector.

34. The method or use according to any one of claims 29, 30 or 32, wherein the said medical condition related to obesity is selected from obesity, NIDDM, hypertension and hyperlipidemia.

35. A method for identifying an agent useful for the treatment of a medical condition related to obesity, said method comprising the steps
(i) identifying a target polypeptide interacting with the FOXC2 polypeptide;
(ii) contacting a candidate agent with the said target polypeptide; and

63

(iii) determining whether said candidate agent stimulates the biological activities of the FOXC2 polypeptide, such stimulation being indicative for a compound useful for the treatment of a medical condition related to obesity.

36. The method according to claim 35 wherein the said stimulation in step (iii) is determined as an increased expression of the FOXC2 polypeptide.

37. The method according to claim 35 wherein the said stimulation in step (iii) is determined as a reduction of total lipid content in a test animal.

38. The method according to any one of claims 35 to 37 wherein the said target polypeptide interacting with the FOXC2 polypeptide is identified using a yeast two-hybrid assay.

Fig. 1

2/17
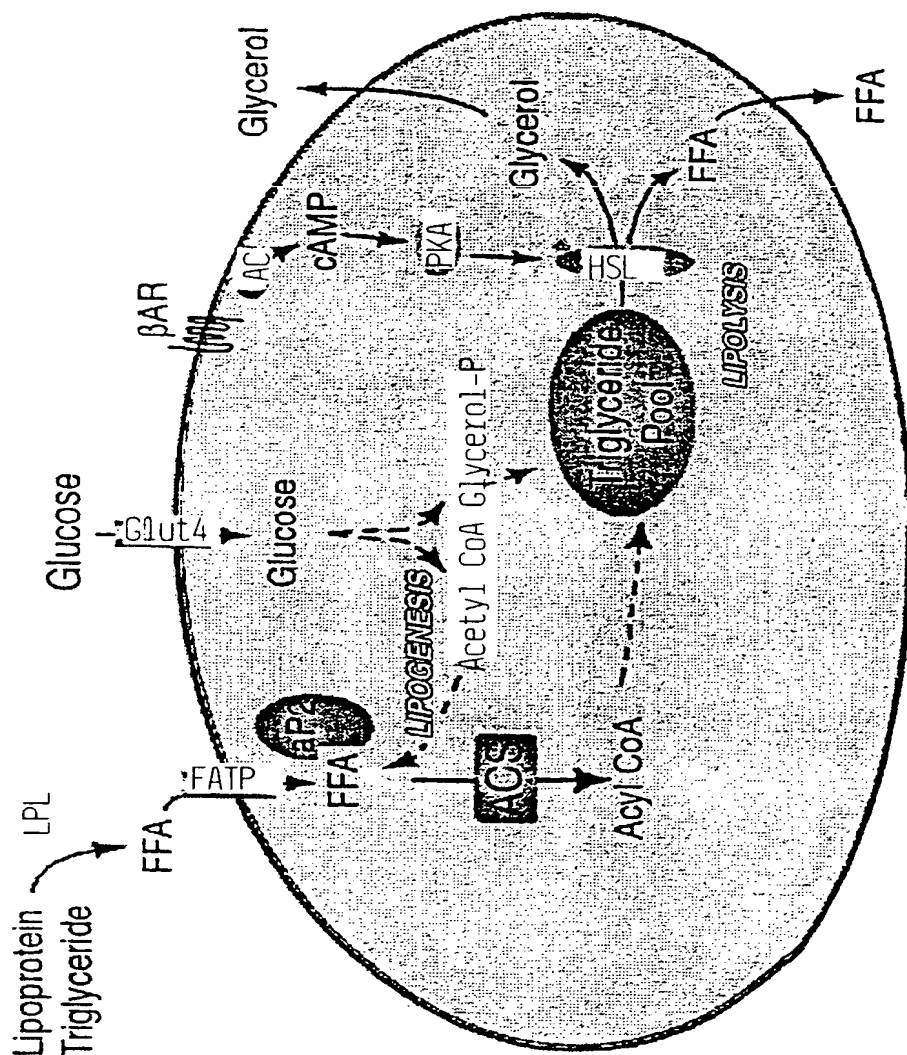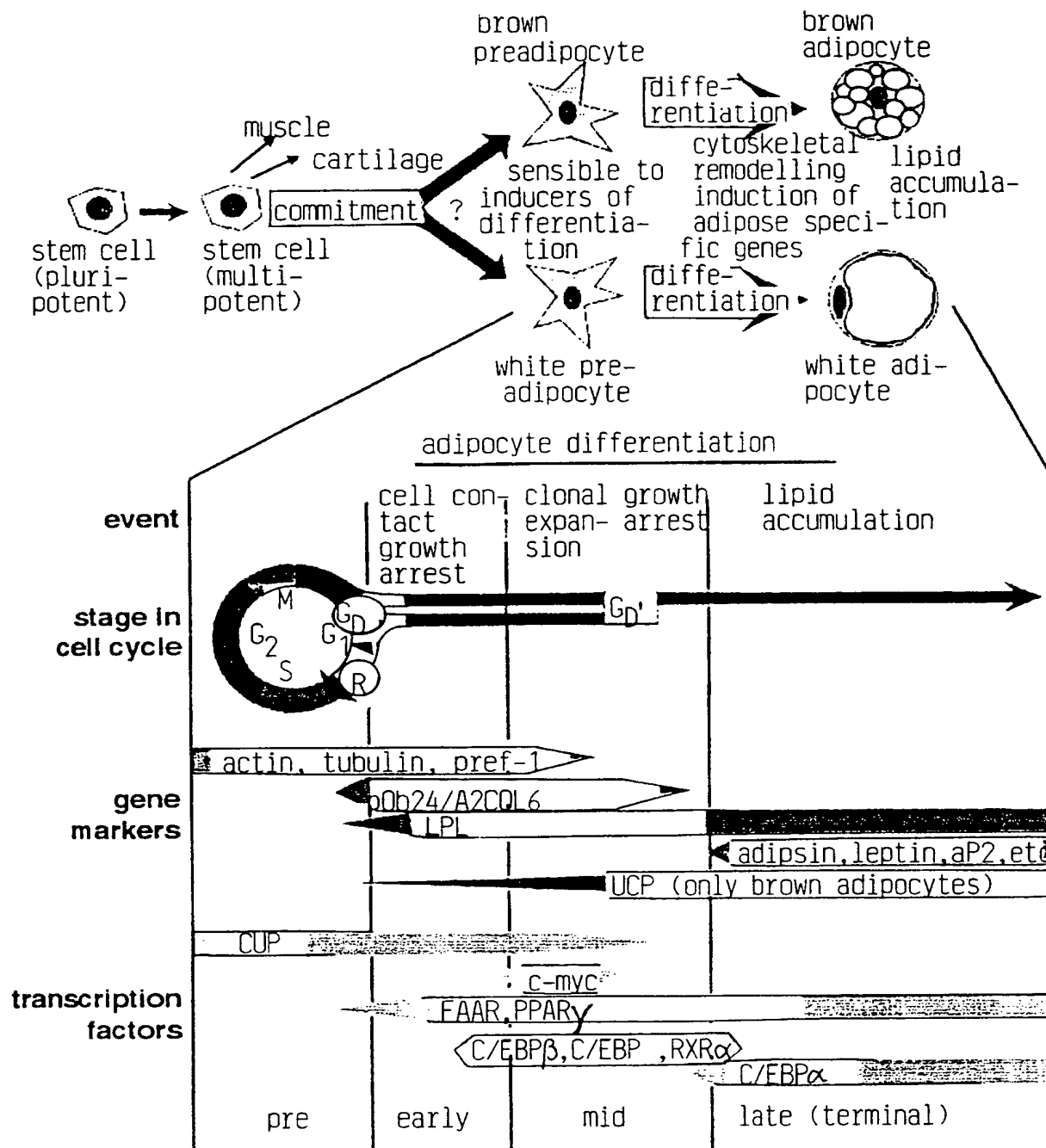
FIG.2
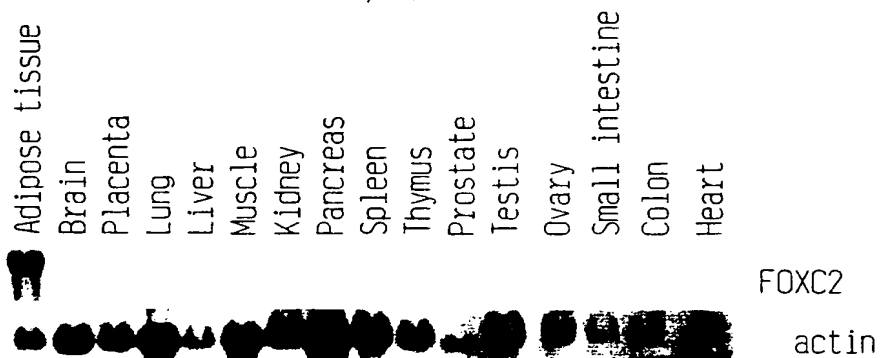
FIG.3a

FIG.3b

FIG.3c

FIG.3d
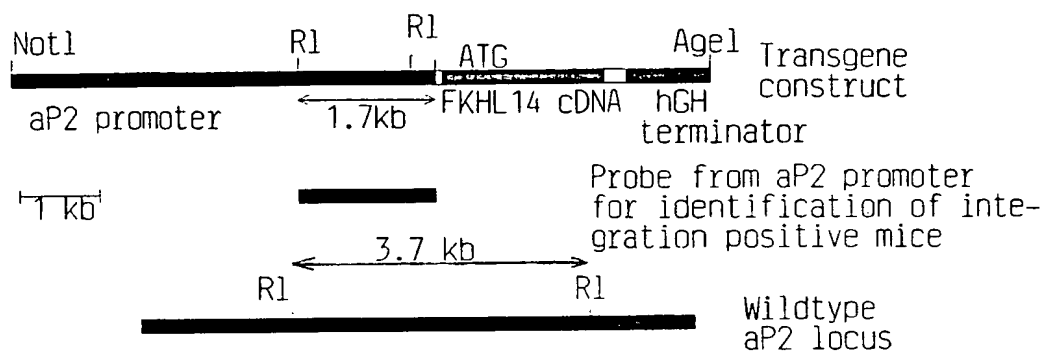
SUBSTITUTE SHEET (RULE 26)

FIG.4a

FIG.4b
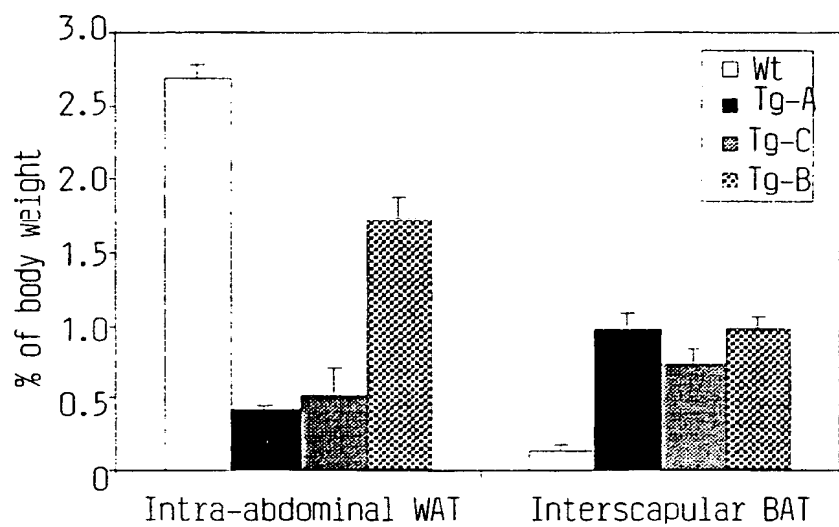
FIG.5a
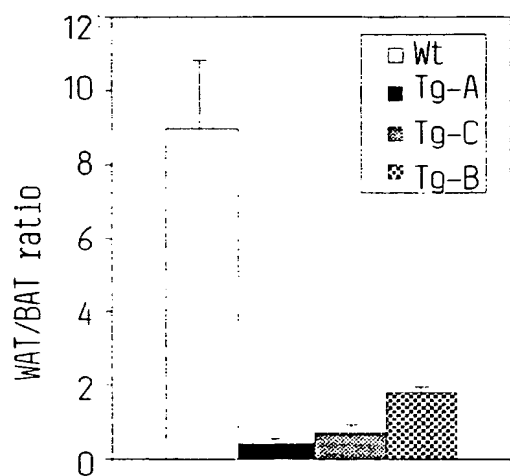
FIG.5b
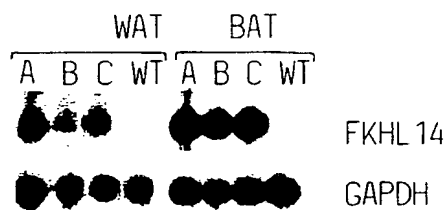
FIG.5c
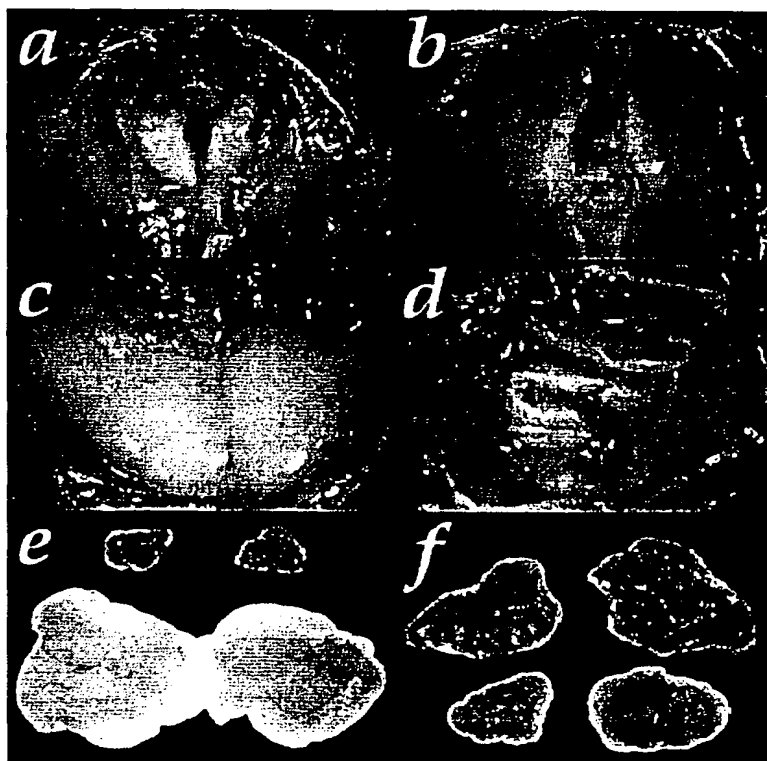
FIG. 6

FIG. 7
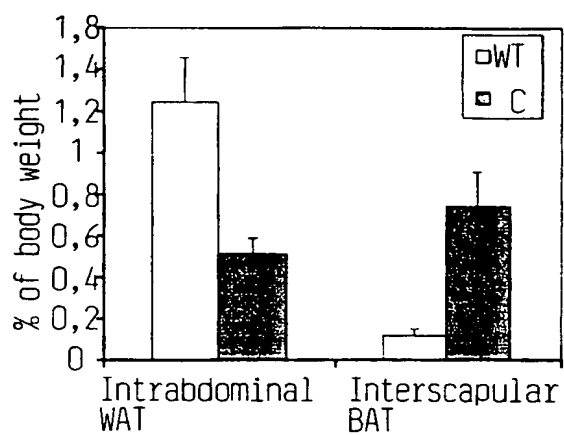
FIG.8a
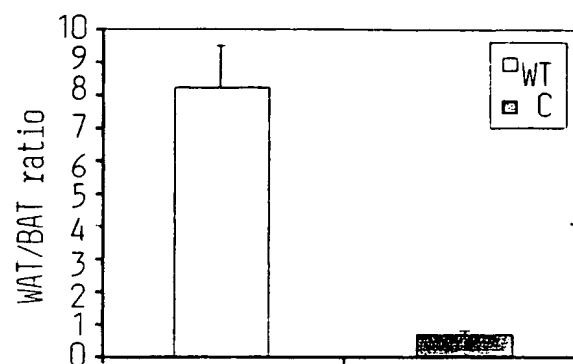
FIG.8b
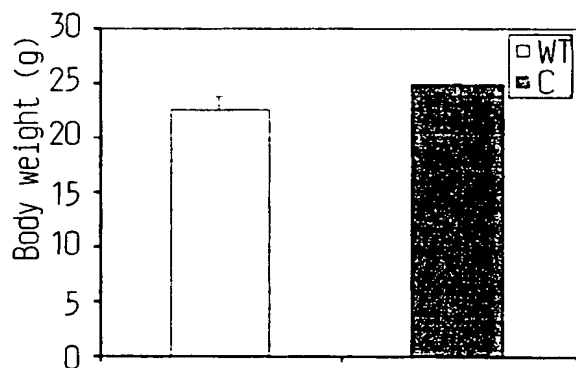
FIG.8c
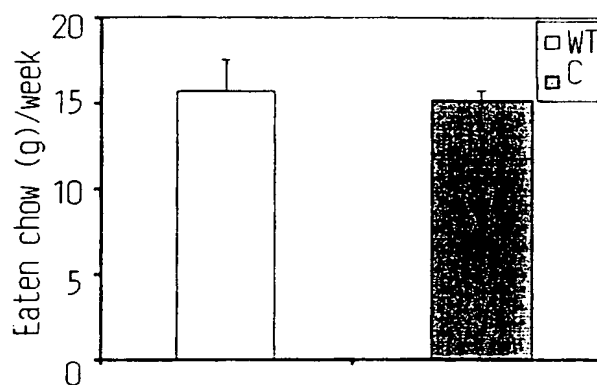
FIG.8d
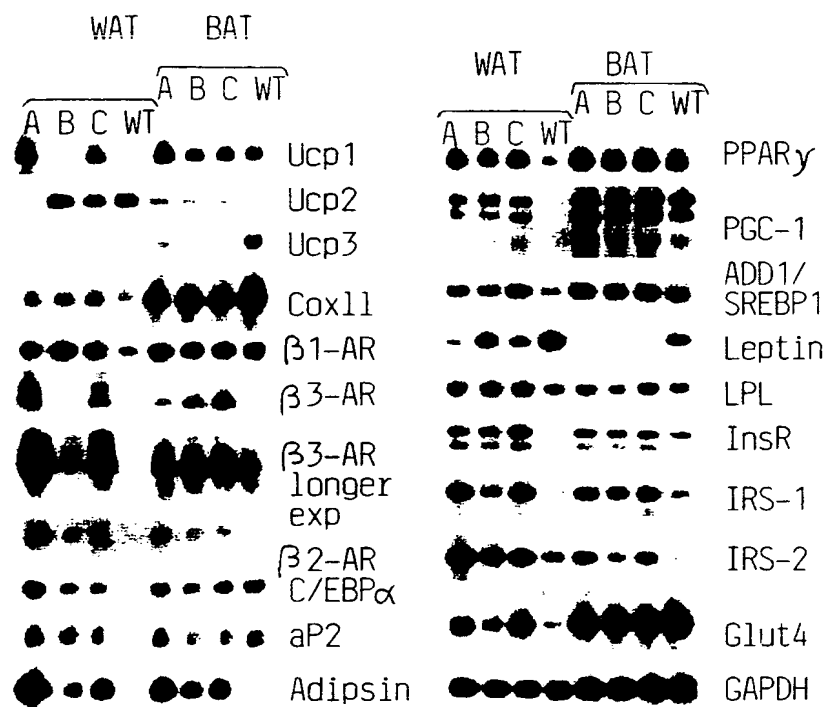
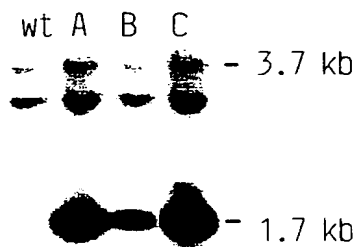FIG.9
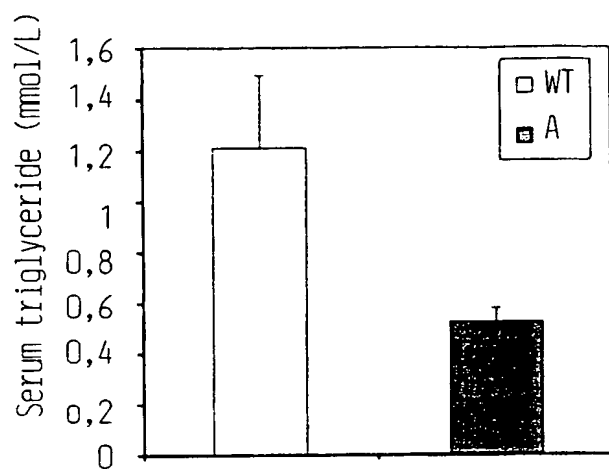
FIG.10
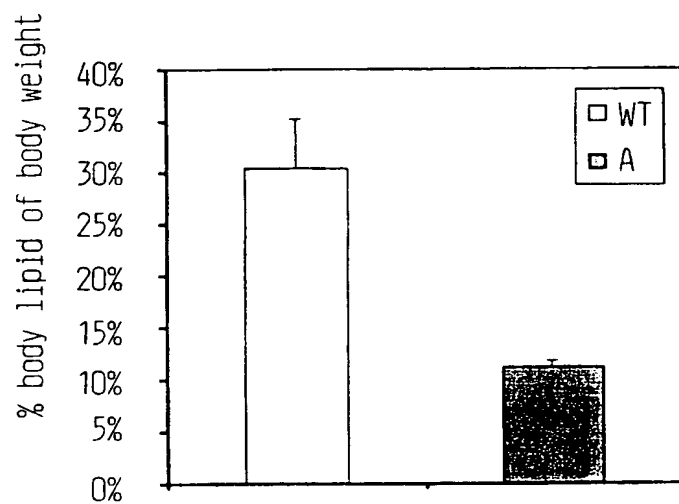
FIG.11

11/17

FIG.12a

FIG.12b

12/17
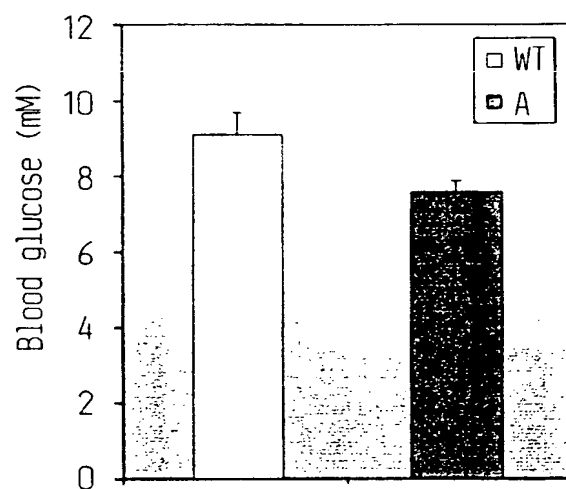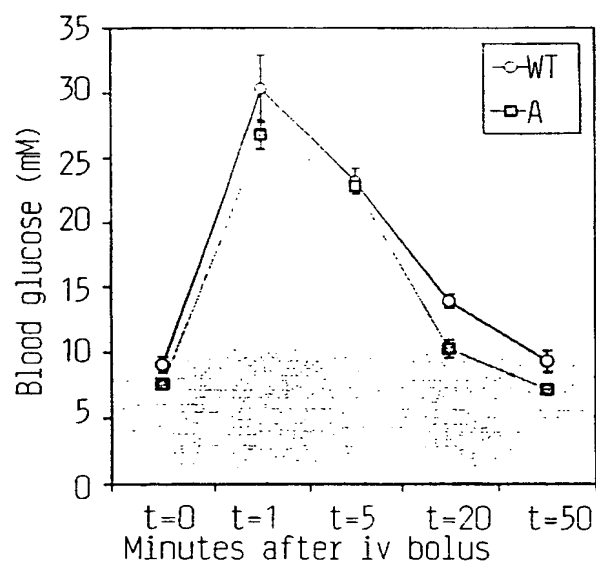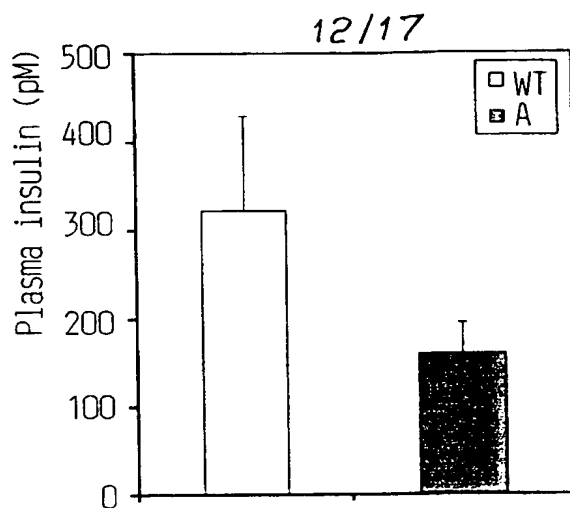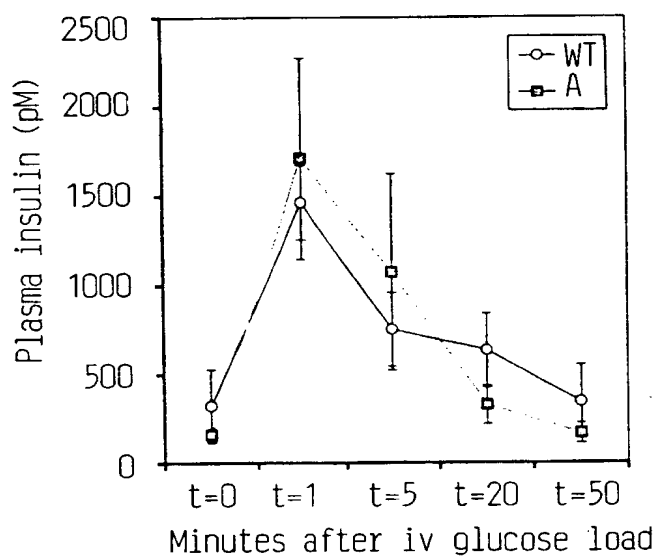
FIG.13A

FIG.13B

FIG.13C

SUBSTITUTE SHEET (RULE 26)

FAS, LPL
Acetyl CoA carboxylase

FATP, ACS
aP2, LPL, PEPCK

ADD1/SREBP1 → PPARγ

FKHL14

Ob, LPL,
Adipsin,
β3-AR, UCP1

C/EBPα

GLUT4, aP2, UCP1, InsR, IRS-1, PEPCK
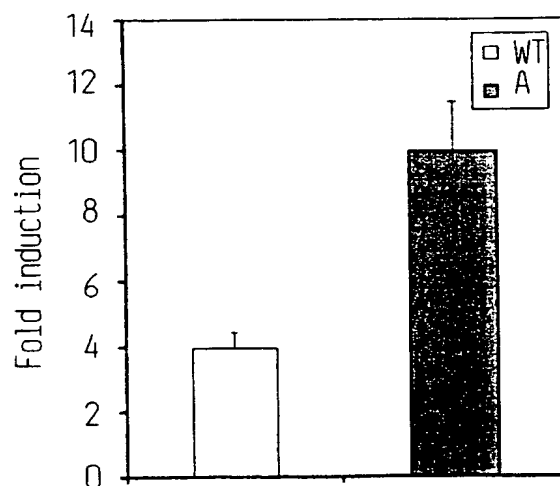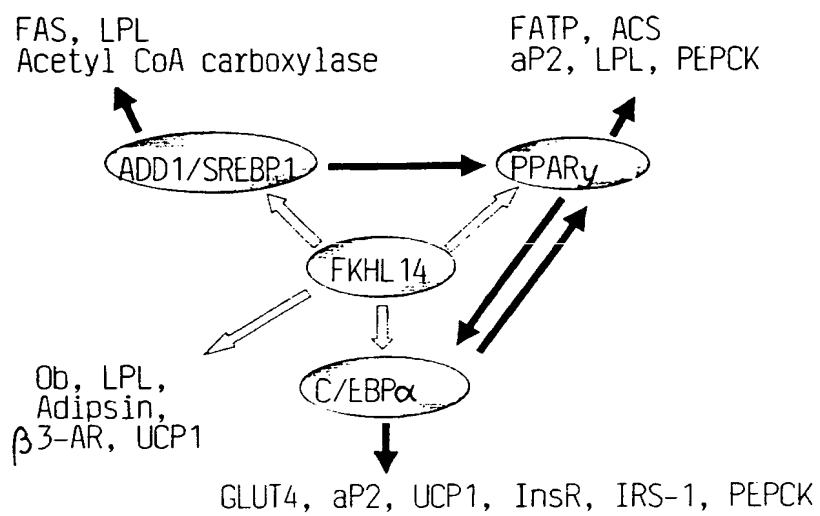
FIG.14
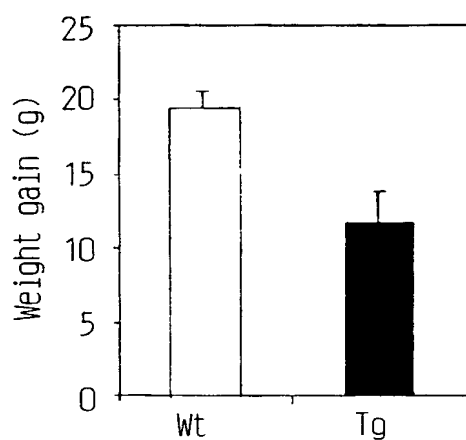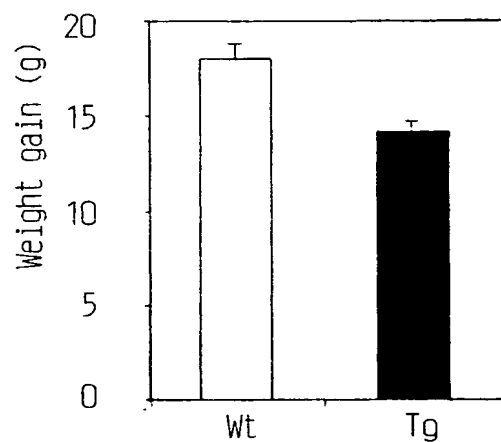
FIG.15a

FIG.15b

SUBSTITUTE SHEET (RULE 26)
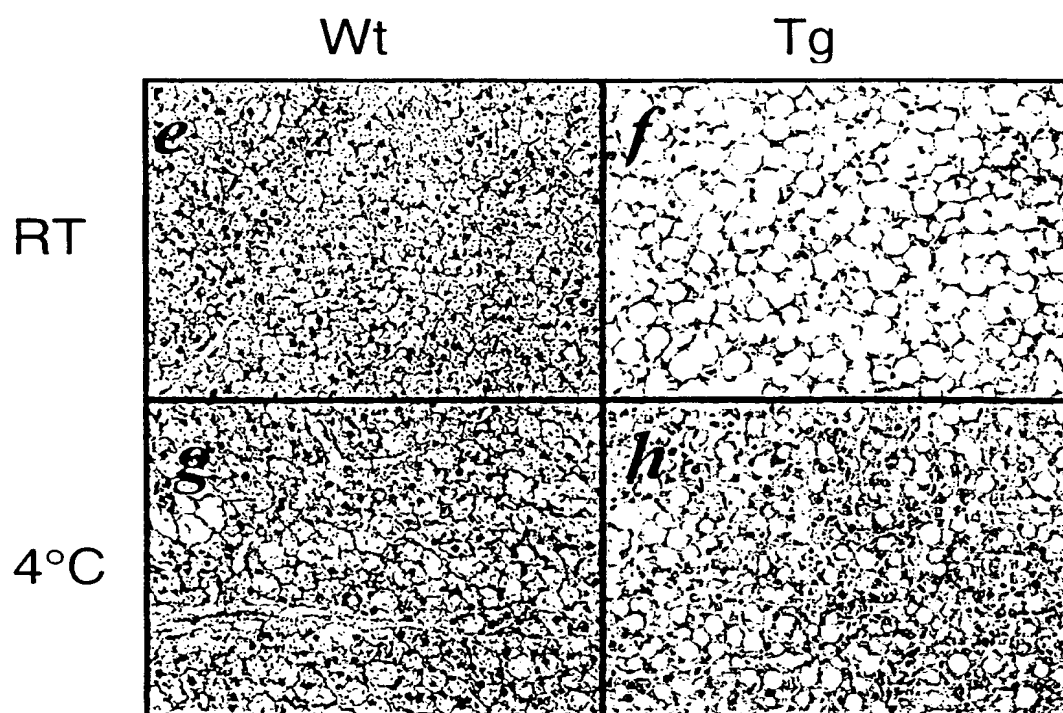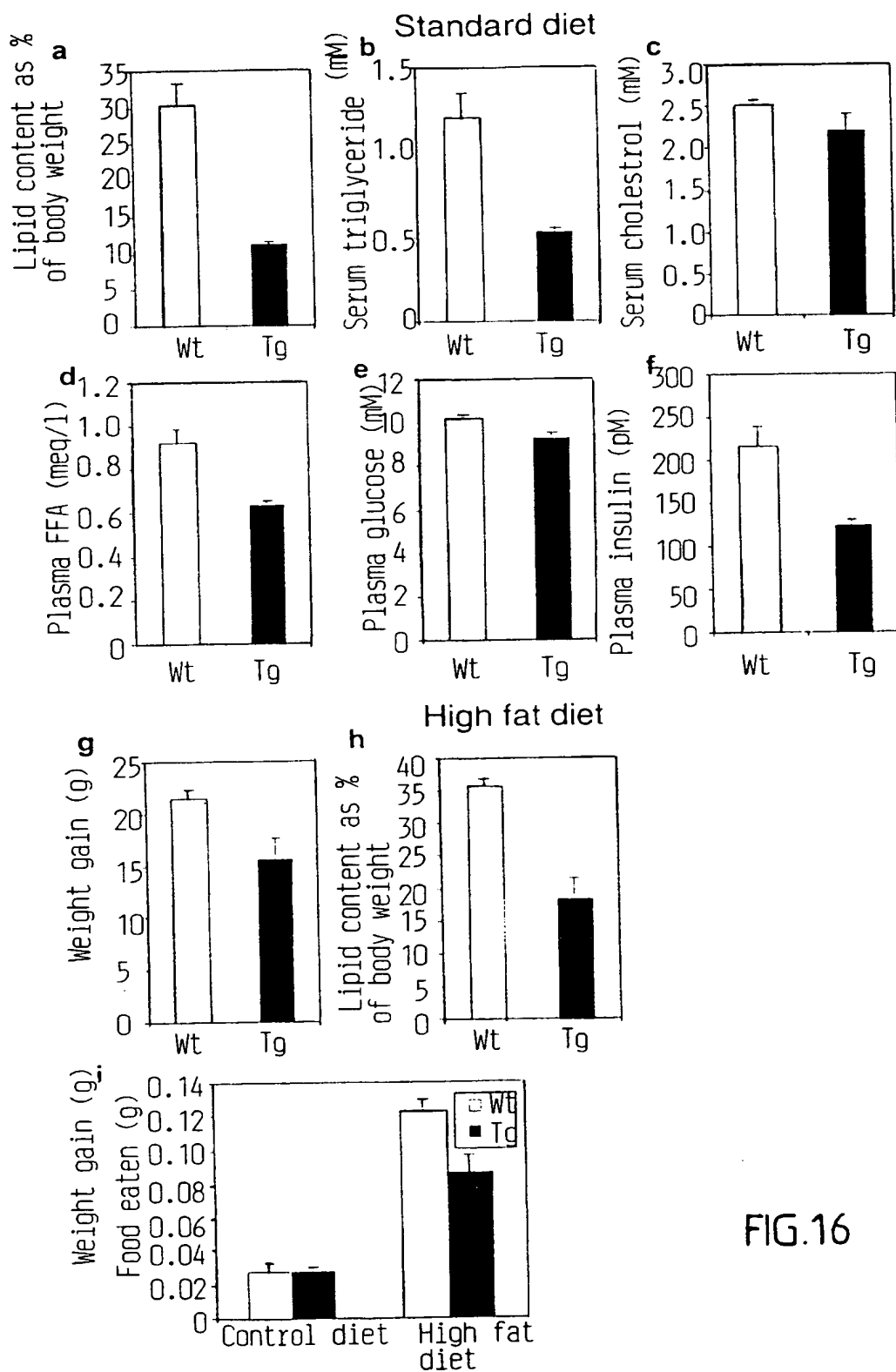
FIG.16
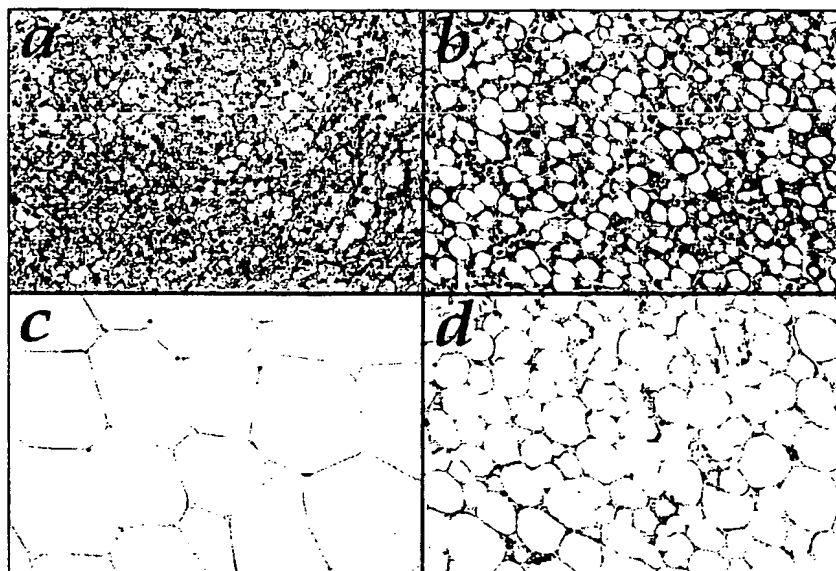
15/17

Standard diet
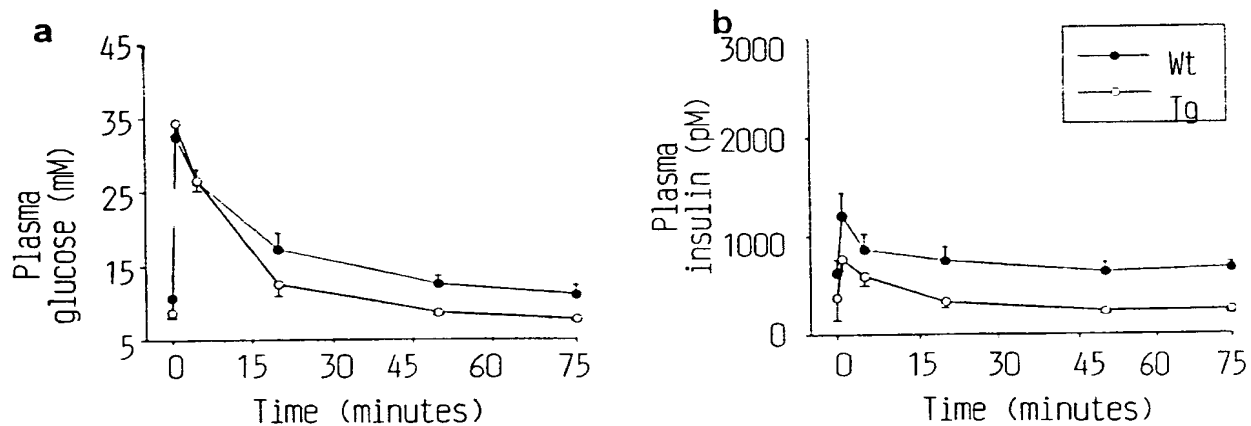
High fat diet

Fig. 17
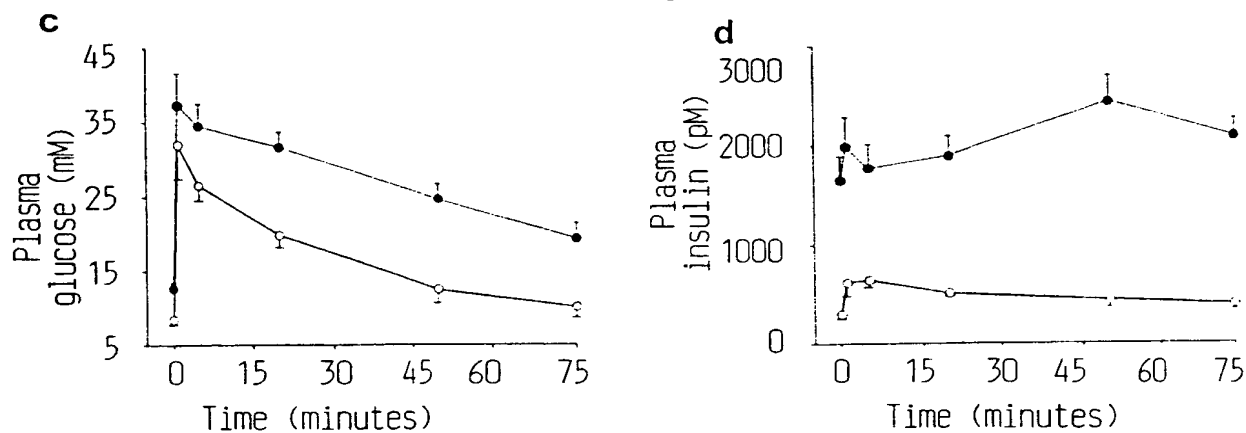
SUBSTITUTE SHEET (RULE 26)
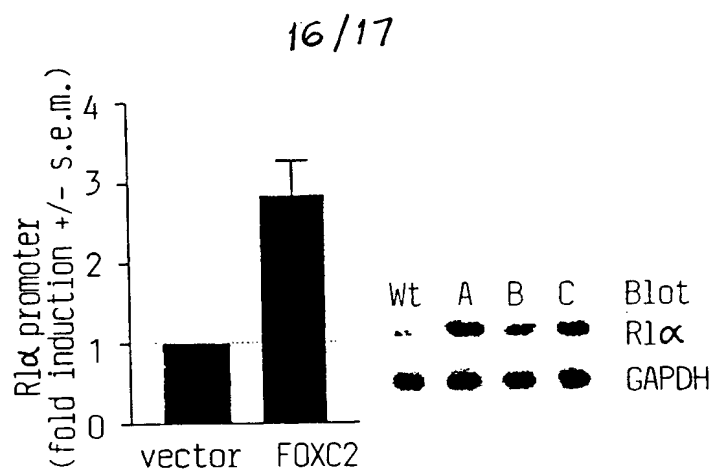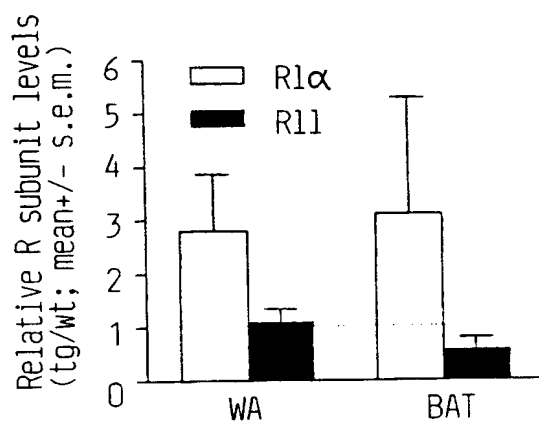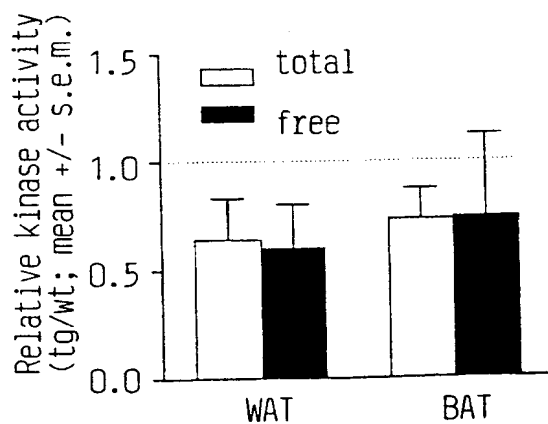
FIG.18a

FIG.18b

FIG.18c

SUBSTITUTE SHEET (RULE 26)
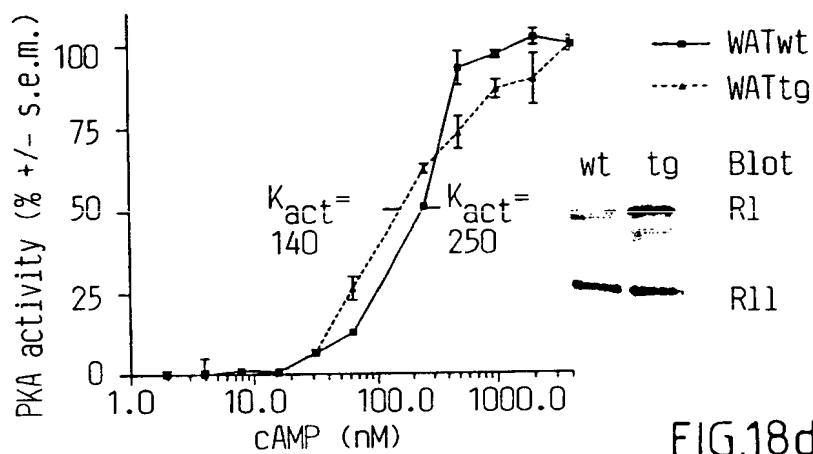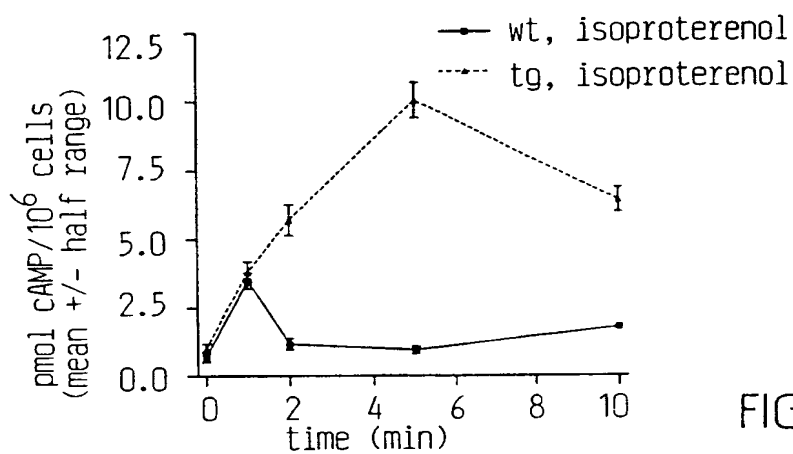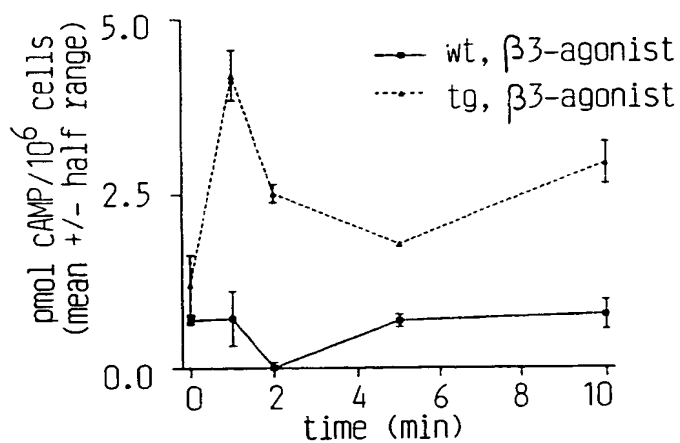
FIG.18d

FIG.18e

FIG.18f

SUBSTITUTE SHEET (RULE 26)

SEQUENCE LISTING

<110> Pharmacia AB

<120> Animal Model

<130> 00083

<140>
<141>

<160> 2

<170> PatentIn Ver. 2.1

<210> 1
<211> 3289
<212> DNA
<213> Homo sapiens

<220>
<221> CDS
<222> (1197)..(2702)

<300>
<308> GenBank/Y08223
<309> 1997-05-14
<313> 1 TO 3289

<300>
<301> Miura et al.,
<303> Genomics
<304> 41
<306> 489-492
<307> 1997

<400> 1
gaattcggag gattaagttg tcagtcagca cgttgctacc ttcccctcta tgcactccgc 60

tgcctggctc ctcggcgggg agcgagggaa actcagtttg tagggtttac ctctaaaacc 120

tcgataggtt atccttgacg accccgagcc tggaaactcc ctgttgatga ttaattattt 180

gattaaataa gtataacatc caggagaggc cctgccattc caatccagcg cgtttgcttt 240

tgaatccatt acacctgggc ccccataatt aggaaatcta attattcgct tcatcactca 300

ttaataagaa aaatgtccca ggatcattgc tacttacaag gtctttggga gagatatttt 360

actctattaa tccattctat tttatatttc aaattgattt ttttttaacag aggaaagtgg 420

ctatcttttt gttttgggca tgtgggccca ttcaccaaaa tgtgatcata aaataaattt 480

taataagata taacttttta aaaagttttc aagtgaagac ggagtcgccg cggaggccgg 540

ggcggcgggg tcttagagcc gacggattcc tgcgctcctc gccccgattg gcgccggact 600

cctctcagct gccgggtgat tggctcaaag ttccgggagg gggcgtggcc cgaggaaagt 660

aaaaactcgc tttcagcaag aagactttg aaacttttcc caatccctaa aagggacttg 720

1

```
gcctcttttt ctgggctcag cggggcagcc gctcggaccc cggcgcgctg accctcgggg 780

ctgccgattc gctggggggct tggagagcct cctgcgcccc tcctcgcgcg ggccgagggt 840

ccaccttggt ccccaggccg cggcgtctcc gctgggtccg cggccgcccg cctgccgcg  900

ctgccgccgc cgggtcctgg agccagcgag gagcggggcc ggcgctgcgc ttgcccgggg 960

cgcgccctcc aggatgccga tccgcccggt ccgctgaaag cgcgcgcccc tgctcggccc 1020

gagcgacgac gaccgcgcac cctcgccccg gaggctgcca ggagaccggg gccgcccctc 1080

ccgctcccct cctctcccccc tctggctctc tcgcgctctc tcgctctcag ggcccccctc 1140

gctcccccgg ccgcagtccg tgcgcgaggg cgccggcgag ccgtctcgga agcagc atg 1199
                                                          Met
                                                           1
```

```
cag gcg cgc tac tcc gtg tcc gac ccc aac gcc ctg gga gtg gtg ccc   1247
Gln Ala Arg Tyr Ser Val Ser Asp Pro Asn Ala Leu Gly Val Val Pro
             5               10               15

tac ctg agc gag cag aat tac tac cgg gct gcg ggc agc tac ggc ggc   1295
Tyr Leu Ser Glu Gln Asn Tyr Tyr Arg Ala Ala Gly Ser Tyr Gly Gly
             20               25               30

atg gcc agc ccc atg ggc gtc tat tcc ggc cac ccg gag cag tac agc   1343
Met Ala Ser Pro Met Gly Val Tyr Ser Gly His Pro Glu Gln Tyr Ser
         35               40               45

gcg ggg atg ggc cgc tcc tac gcg ccc tac cac cac cac cag ccc gcg   1391
Ala Gly Met Gly Arg Ser Tyr Ala Pro Tyr His His His Gln Pro Ala
     50               55               60               65

gcg cct aag gac ctg gtg aag ccg ccc tac agc tac atc gcg ctc atc   1439
Ala Pro Lys Asp Leu Val Lys Pro Pro Tyr Ser Tyr Ile Ala Leu Ile
             70               75               80

acc atg gcc atc cag aac gcg ccc gag aag aag atc acc ttg aac ggc   1487
Thr Met Ala Ile Gln Asn Ala Pro Glu Lys Lys Ile Thr Leu Asn Gly
                 85               90               95

atc tac cag ttc atc atg gac cgc ttc ccc ttc tac cgg gag aac aag   1535
Ile Tyr Gln Phe Ile Met Asp Arg Phe Pro Phe Tyr Arg Glu Asn Lys
         100              105              110

cag ggc tgg cag aac agc atc cgc cac aac ctc tcg ctc aac gag tgc   1583
Gln Gly Trp Gln Asn Ser Ile Arg His Asn Leu Ser Leu Asn Glu Cys
     115              120              125

ttc gtc aag gtg ccc cgc gac gac aag aag ccc ggc aag ggc agt tac   1631
Phe Val Lys Val Pro Arg Asp Asp Lys Lys Pro Gly Lys Gly Ser Tyr
130              135              140              145

tgg acc ctg gac ccg gac tcc tac aac atg ttc gag aac ggc agc ttc   1679
Trp Thr Leu Asp Pro Asp Ser Tyr Asn Met Phe Glu Asn Gly Ser Phe
             150              155              160

ctg cgg cgc cgg cgg cgc ttc aaa aag aag gac gtg tcc aag gag aag   1727
Leu Arg Arg Arg Arg Arg Phe Lys Lys Lys Asp Val Ser Lys Glu Lys
                 165              170              175
```

2
```

```
gag gag cgg gcc cac ctc aag gag ccg ccc ccg gcg gcg tcc aag ggc    1775
Glu Glu Arg Ala His Leu Lys Glu Pro Pro Pro Ala Ala Ser Lys Gly
            180                 185                 190

gcc ccg gcc acc ccc cac cta gcg gac gcc ccc aag gag gcc gag aag    1823
Ala Pro Ala Thr Pro His Leu Ala Asp Ala Pro Lys Glu Ala Glu Lys
        195                 200                 205

aag gtg gtg atc aag agc gag gcg gcg tcc ccg gcg ctg ccg gtc atc    1871
Lys Val Val Ile Lys Ser Glu Ala Ala Ser Pro Ala Leu Pro Val Ile
210                 215                 220                 225

acc aag gtg gag acg ctg agc ccc gag agc gcg ctg cag ggc agc ccg    1919
Thr Lys Val Glu Thr Leu Ser Pro Glu Ser Ala Leu Gln Gly Ser Pro
                230                 235                 240

cgc agc gcg gcc tcc acg ccc gcc ggc tcc ccc gac ggt tcg ctg ccg    1967
Arg Ser Ala Ala Ser Thr Pro Ala Gly Ser Pro Asp Gly Ser Leu Pro
            245                 250                 255

gag cac cac gcc gcg gcg ccc aac ggg ctg cct ggc ttc agc gtg gag    2015
Glu His His Ala Ala Ala Pro Asn Gly Leu Pro Gly Phe Ser Val Glu
            260                 265                 270

aac atc atg acc ctg cga acg tcg ccg ccg ggc gga gag ctg agc ccg    2063
Asn Ile Met Thr Leu Arg Thr Ser Pro Pro Gly Gly Glu Leu Ser Pro
        275                 280                 285

ggg gcc gga cgc gcg ggc ctg gtg gtg ccg ccg ctg gcg ctg cca tac    2111
Gly Ala Gly Arg Ala Gly Leu Val Val Pro Pro Leu Ala Leu Pro Tyr
290                 295                 300                 305

gcc gcc gcg ccg ccc gcc gcc tac ggc cag ccg tgc gct cag ggc ctg    2159
Ala Ala Ala Pro Pro Ala Ala Tyr Gly Gln Pro Cys Ala Gln Gly Leu
            310                 315                 320

gag gcc ggg gcc gcc ggg ggc tac cag tgc agc atg cga gcg atg agc    2207
Glu Ala Gly Ala Ala Gly Gly Tyr Gln Cys Ser Met Arg Ala Met Ser
            325                 330                 335

ctg tac acc ggg gcc gag cgg ccg gcg cac atg tgc gtc ccg ccc gcc    2255
Leu Tyr Thr Gly Ala Glu Arg Pro Ala His Met Cys Val Pro Pro Ala
            340                 345                 350

ctg gac gag gcc ctc tcg gac cac ccg agc ggc ccc acg tcg ccc ctg    2303
Leu Asp Glu Ala Leu Ser Asp His Pro Ser Gly Pro Thr Ser Pro Leu
        355                 360                 365

agc gct ctc aac ctc gcc gcc ggc cag gag ggc gcg ctc gcc gcc acg    2351
Ser Ala Leu Asn Leu Ala Ala Gly Gln Glu Gly Ala Leu Ala Ala Thr
370                 375                 380                 385

ggc cac cac cac cag cac cac ggc cac cac cac ccg cag gcg ccg ccg    2399
Gly His His His Gln His His Gly His His His Pro Gln Ala Pro Pro
                390                 395                 400

ccc ccg ccg gct ccc cag ccc cag ccg acg ccg cag ccc ggg gcc gcc    2447
Pro Pro Pro Ala Pro Gln Pro Gln Pro Thr Pro Gln Pro Gly Ala Ala
            405                 410                 415
```

3

```
gcg gcg cag gcg gcc tcc tgg tat ctc aac cac agc ggg gac ctg aac    2495
Ala Ala Gln Ala Ala Ser Trp Tyr Leu Asn His Ser Gly Asp Leu Asn
        420             425             430

cac ctc ccc ggc cac acg ttc gcg gcc cag cag caa act ttc ccc aac    2543
His Leu Pro Gly His Thr Phe Ala Ala Gln Gln Gln Thr Phe Pro Asn
        435             440             445

gtg cgg gag atg ttc aac tcc cac cgg ctg ggg att gag aac tcg acc    2591
Val Arg Glu Met Phe Asn Ser His Arg Leu Gly Ile Glu Asn Ser Thr
450             455             460             465

ctc ggg gag tcc cag gtg agt ggc aat gcc agc tgc cag ctg ccc tac    2639
Leu Gly Glu Ser Gln Val Ser Gly Asn Ala Ser Cys Gln Leu Pro Tyr
            470             475             480

aga tcc acg ccg cct ctc tat cgc cac gca gcc ccc tac tcc tac gac    2687
Arg Ser Thr Pro Pro Leu Tyr Arg His Ala Ala Pro Tyr Ser Tyr Asp
            485             490             495

tgc acg aaa tac tga cgtgtcccgg gacctcccct ccccggcccg ctccggcttc    2742
Cys Thr Lys Tyr
        500

gcttcccagc cccgacccaa ccagacaatt aaggggctgc agagacgcaa aaaagaaaca 2802

aaacatgtcc accaaccttt tctcagaccc gggagcagag agcgggcacg ctagccccca 2862

gccgtctgtg aagagcgcag gtaactttaa ttcgccgccc cgtttctggg atcccaggaa 2922

acccctccaa agggacgcag cccaacaaaa tgagtattgg tcttaaaatc cccctcccct 2982

accaggacgg ctgtgctgtg ctcgacctga gctttcaaaa gttaagttat ggacccaaat 3042

cccatagcga gccctagtg actttctgta ggggtcccca taggtgtatg ggggtctcta 3102

tagataatat atgtgctgtg tgtaattta aatttctcca accgtgctgt acaaatgtgt 3162

ggatttgtaa tcaggctatt ttgttgttgt tgttgttgtt cagagccatt aatataatat 3222

ttaaagttga gttcactgga taagttttc atcttgccca accatttcta actgccaaat 3282

tgaattc                                                             3289
```

<210> 2
<211> 501
<212> PRT
<213> Homo sapiens

<400> 2

```
Met Gln Ala Arg Tyr Ser Val Ser Asp Pro Asn Ala Leu Gly Val Val
1               5               10              15
Pro Tyr Leu Ser Glu Gln Asn Tyr Tyr Arg Ala Ala Gly Ser Tyr Gly
        20              25              30
Gly Met Ala Ser Pro Met Gly Val Tyr Ser Gly His Pro Glu Gln Tyr
        35              40              45
Ser Ala Gly Met Gly Arg Ser Tyr Ala Pro Tyr His His Gln Pro
    50              55              60
Ala Ala Pro Lys Asp Leu Val Lys Pro Pro Tyr Ser Tyr Ile Ala Leu
65              70              75              80
```

4

```
Ile Thr Met Ala Ile Gln Asn Ala Pro Glu Lys Lys Ile Thr Leu Asn
              85                      90                  95
Gly Ile Tyr Gln Phe Ile Met Asp Arg Phe Pro Phe Tyr Arg Glu Asn
             100                     105                 110
Lys Gln Gly Trp Gln Asn Ser Ile Arg His Asn Leu Ser Leu Asn Glu
             115                     120                 125
Cys Phe Val Lys Val Pro Arg Asp Asp Lys Lys Pro Gly Lys Gly Ser
    130          ·          135                 140
Tyr Trp Thr Leu Asp Pro Asp Ser Tyr Asn Met Phe Glu Asn Gly Ser
145                  150                 155                 160
Phe Leu Arg Arg Arg Arg Arg Phe Lys Lys Lys Asp Val Ser Lys Glu
                 165                 170                 175
Lys Glu Glu Arg Ala His Leu Lys Glu Pro Pro Pro Ala Ala Ser Lys
             180                 185                 190
Gly Ala Pro Ala Thr Pro His Leu Ala Asp Ala Pro Lys Glu Ala Glu
             195                 200                 205
Lys Lys Val Val Ile Lys Ser Glu Ala Ala Ser Pro Ala Leu Pro Val
    210                 215                 220
Ile Thr Lys Val Glu Thr Leu Ser Pro Glu Ser Ala Leu Gln Gly Ser
225                  230                 235                 240
Pro Arg Ser Ala Ala Ser Thr Pro Ala Gly Ser Pro Asp Gly Ser Leu
             245                 250                 255
Pro Glu His His Ala Ala Ala Pro Asn Gly Leu Pro Gly Phe Ser Val
             260                 265                 270
Glu Asn Ile Met Thr Leu Arg Thr Ser Pro Pro Gly Gly Glu Leu Ser
             275                 280                 285
Pro Gly Ala Gly Arg Ala Gly Leu Val Val Pro Pro Leu Ala Leu Pro
    290                 295                 300
Tyr Ala Ala Ala Pro Pro Ala Ala Tyr Gly Gln Pro Cys Ala Gln Gly
305                  310                 315                 320
Leu Glu Ala Gly Ala Ala Gly Gly Tyr Gln Cys Ser Met Arg Ala Met
             325                 330                 335
Ser Leu Tyr Thr Gly Ala Glu Arg Pro Ala His Met Cys Val Pro Pro
             340                 345                 350
Ala Leu Asp Glu Ala Leu Ser Asp His Pro Ser Gly Pro Thr Ser Pro
    355                 360                 365
Leu Ser Ala Leu Asn Leu Ala Ala Gly Gln Glu Gly Ala Leu Ala Ala
    370                 375                 380
Thr Gly His His His Gln His His Gly His His His Pro Gln Ala Pro
385                  390                 395                 400
Pro Pro Pro Pro Ala Pro Gln Pro Gln Pro Thr Pro Gln Pro Gly Ala
             405                 410                 415
Ala Ala Ala Gln Ala Ala Ser Trp Tyr Leu Asn His Ser Gly Asp Leu
             420                 425                 430
Asn His Leu Pro Gly His Thr Phe Ala Ala Gln Gln Gln Thr Phe Pro
    435                 440                 445
Asn Val Arg Glu Met Phe Asn Ser His Arg Leu Gly Ile Glu Asn Ser
    450                 455                 460
Thr Leu Gly Glu Ser Gln Val Ser Gly Asn Ala Ser Cys Gln Leu Pro
465                  470                 475                 480
Tyr Arg Ser Thr Pro Pro Leu Tyr Arg His Ala Ala Pro Tyr Ser Tyr
             485                 490                 495
Asp Cys Thr Lys Tyr
             500
```

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE 01/00339

A. CLASSIFICATION OF SUBJECT MATTER

IPC7: C07K 14/435, A61K 38/17, A01K 67/027

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC7: C07K, A61K, A01K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 9854216 A1 (PHARMACIA & UPJOHN AB), 3 December 1998 (03.12.98)	1-2,4-16, 20-25-29, 31-38
Y		3
	--	
Y	WO 9206104 A1 (THE DANA-FARBER CANCER INSTITUTE), 16 April 1992 (16.04.92), page 24, line 13 - line 23, claim 20	3
A		1-2,4-16, 20-25,29, 31-38
	--	

[X] Further documents are listed in the continuation of Box C.   [X] See patent family annex.

*	Special categories of cited documents:
"A"	document defining the general state of the art which is not considered to be of particular relevance
"E"	earlier application or patent but published on or after the international filing date
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O"	document referring to an oral disclosure, use, exhibition or other means
"P"	document published prior to the international filing date but later than the priority date claimed

"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X"	document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y"	document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&"	document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
2 July 2001	0 2 -07- 2001

| Name and mailing address of the ISA:
Swedish Patent Office
Box 5055, S-102 42 STOCKHOLM
Facsimile No. +46 8 666 02 86 | Authorized officer

Patrick Andersson/BS
Telephone No. +46 8 782 25 00 |

Form PCT ISA/210 (second sheet) (July 1998)

INTERNATIONAL SEARCH REPORT

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Genomics, Volume 41, 1997, Naoyuki Miura et al, "Isolation of the Mouse (MFH-1) and Human (FKHL14) Mesenchyme Fork Head-1 Genes Reveals Conversation of Their Gene and Protein Structures" page 489 - page 492	1,4,31
A		2-3,5-16, 20-25,29, 31-38
	--	
A	WO 9952415 A1 (THE UNIVERSITY OF IOWA RESEARCH FOUNDATION), 21 October 1999 (21.10.99), page 9, line 11 - line 17; page 1, line 28 - line 33	1-16,20-25, 29,31-38
	-- --------	

INTERNATIONAL SEARCH REPORT

Box I	Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: **18-19, 27-28, 30 and 32-34**
   because they relate to subject matter not required to be searched by this Authority, namely:

   see next sheet*

2. ☒ Claims Nos.: **claims 17-19 and 26-28**
   because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

   see next sheet**

3. ☐ Claims Nos.:
   because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II	Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see next sheet***

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest
☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet (1)) (July1998)